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

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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$_{β}$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$_{α}$A1 with retinoid X receptor did not enhance regulation. Deletion of the DNA binding domain and the nuclear localization signal of the TR$_{α}$A1 eliminated transcriptional activity but did not affect the ability to regulate Ca$^{2+}$ signaling. T$_3$-bound TR$_{β}$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$_{β}$A1 and the homologous shortened form of rat TR$_{α}$ (rTR$_{α}$AF1) localized to the mitochondria and increased O$_2$ consumption, whereas the full-length rat TR$_{α}$ did neither. Furthermore, only T$_3$-bound xTR$_{β}$A1 and rTR$_{α}$AF1 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 (Abbatichio 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

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Abbreviations used in this paper: ANT, adenine nucleotide translocator; DBD, DNA binding domain; ΔΨ, 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$_{β}$, rat thyroid hormone receptor alpha subtype 1; rTR$_{α}$AF1, shortened form of rat TR$_{α}$; Ru$_{3900}$, ruthenium 3900; 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$_{β}$A1, Xenopus thyroid hormone receptor beta subtype A1.

Increased evidence suggests that thyroid hormone exerts nontranscriptional effects on mitochondrial metabolism. Initial studies demonstrated that treatment of cells with 3,5,3’-tri-iodothyronine (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-
transcriptional. Sterling and coworkers (Sterling, 1980; Sterling and Brenner, 1995) additionally demonstrated that exposure of mitochondria to T3, isolated from rat hepatocytes, increased both ATP production and O2 consumption. Acute exposure of isolated mitochondria to thyroid hormone has also been reported to increase ΔpH and to increase mitochondrial Ca2+ influx (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 T3 binding proteins in rat liver mitochondria. Wrutniak et al. (1995) and Casas et al. (1999) reported the presence of a high affinity (~43 kD) T3 binding protein in rat liver mitochondrial matrix extracts, which was identified as an NH2 terminus shortened form of rat TRA1 (rTRΔF1). The full-length form of the rat thyroid hormone receptor alpha subtype 1 (rTRα1) 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 rTRs may be involved in mitochondrial transcriptional activity.

Intracellular Ca2+ signaling has been intimately linked to mitochondrial metabolism. Several dehydrogenases within the citric acid cycle are Ca2+ dependent (McCormack and Denton, 1989). Ca2+ uptake into the mitochondria is a passive process driven by the mitochondrial ΔΨ and occurs via the Ca2+ uniporter. Because of the low Ca2+ affinity of the uniporter, high cytosolic Ca2+ concentrations are required to cause significant mitochondrial Ca2+ 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 Ca2+ uptake (Rizzuto et al., 1998, 1999). Work from our laboratory also demonstrated that mitochondrial Ca2+ uptake itself modulated inositol 1,4,5-trisphosphate (IP3)-Ca2+ release (Jouaville et al., 1995). Subsequently, Hajnoczky et al. (1995) demonstrated that IP3-mediated Ca2+ oscillations efficiently stimulated mitochondrial metabolism. The local Ca2+ 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 Ca2+ 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 Ca2+ signaling and mitochondrial metabolism.

We present evidence demonstrating that thyroid hormone-activated TRs acutely regulate mitochondrial metabolism and, thereby, Ca2+ wave activity. Only expression of the NH2 terminus–truncated forms of TR that target the mitochondria were effective at stimulating mitochondria. Transcriptionally inactive TRs were fully capable of modulating Ca2+ wave activity. These observations suggest an acute nontranscriptional pathway for modulation of intracellular Ca2+ signaling via thyroid hormone receptor-stimulated mitochondrial metabolism.

Results

T3-stimulated TRA1s modulate IP3-mediated Ca2+ 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 Ca2+ signaling, stage VI Xenopus oocytes were injected with mRNA encoding the Xenopus thyroid hormone receptor betz subtype A1 (xTRA1) as described previously (Camacho and Lechleiter, 2000). Expression of xTRA1 was confirmed by Western blot analysis, 2–3 d after mRNA injection (Fig. 1). The Ca2+ indicator dye was injected into oocytes 30–45 min before confocal imaging. When oocytes were injected with IP3, we observed repetitive Ca2+ wave activity with interwave periods of 6.62 ± 0.20 s (n = 70; Fig. 1). When xTRA1 expressing oocytes were treated with T3 10 min before IP3 injection, the Ca2+ wave periodicity increased significantly to 8.40 ± 0.30 s (Fig. 1, a and c; n = 24, P < 0.0001 ANOVA single factor). Treatment of oocytes with T3 by itself did not induce Ca2+ release and no detectable changes in basal intracellular Ca2+ concentrations were observed. Application of T3 ligand to nonexpressing control oocytes, had no effect on the

![Figure 1](image_url)
Similarly, xTR also significantly increased SEAP immunoreactivity, which was present only in oocytes expressing xTR. Oocytes expressing xTR or TR plus xRXR, were incubated with 100 nM T3 (lanes 3–5) plus 100 nM RA (lane 5) for 3 d. Cytosolic extracts from each group of oocytes were 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 xTR and xRXR, exposed to both T3 and RA. (b) Transcriptional activity of TRA1 requires both ligands. Oocytes expressing TRA1 and xRXR, were incubated with T3 (100 nM) and RA (100 nM) for 3 d, SEAP expression was significantly increased (Fig. 2 a, lane 5). Note that xTR-mediated transcription requires both ligands, T3 and RA (Fig. 2 a, lanes 4 and 5). These data indicate that stimulation of xTR by T3 does not initiate detectable transcription in Xenopus oocytes.

\[ \text{Ca}^{2+} \text{ interwave period (6.38 ± 0.34 s, } n = 27, \text{ Fig. 1, a and c).} \]

Similarly, xTRpA1 expressing oocytes without T3 treatment exhibited no change in Ca2+ wave periodicity (6.97 ± 0.24 s, n = 35). Peak Ca2+ wave amplitudes (ΔF/F) for xTRpA1-expressing oocytes exposed to T3 (0.69 ± 0.04, n = 29) was also significantly higher than that of control oocytes exposed to T3 (0.55 ± 0.04, n = 20; P < 0.05, t test). We conclude from these data that T3-stimulated xTRpA1 acutely modulates IP3-mediated Ca2+ wave activity and that both thyroid hormone and receptor expression are required for these effects.

**Transcriptional activity of TRA1 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, we coinjected oocytes with xTR and mRNA and a plasmid reporting vector containing a TRE system with two direct repeats (DR4) upstream of the secreted placentai 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 T3 (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 protein by itself (Fig. 2 a, lane 3). However, when we coexpressed xRXRs with xTR and oocytes were incubated with T3 (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 xTR1/xRXR-mediated transcription requires both ligands, T3 and RA (Fig. 2 a, lanes 4 and 5). These data indicate that stimulation of xTR by T3 does not initiate detectable transcription in Xenopus oocytes.

**Acute modulation of Ca2+ signaling does not require heterodimerization with RXR**

To test whether heterodimerization of xTR with xRXR affects the acute modulation of Ca2+ activity, we coinjected oocytes with both xRXR and xTR and mRNA and confirmed protein expression levels using Western analysis 2–3 d after injection of mRNA (Fig. 3 b). Oocytes were loaded with Ca2+ indicator dye and confocally imaged. Oocytes coexpressing xTR and xRXR were incubated with T3 (100 nM) and RA (100 nM) for 3 d, SEAP expression was significantly increased (Fig. 2 a, lane 5). Note that xTR1/xRXR-mediated transcription requires both ligands, T3 and RA (Fig. 2 a, lanes 4 and 5). These data indicate that stimulation of xTR by T3 does not initiate detectable transcription in Xenopus oocytes.

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**Figure 2. Transcriptional activity of TRA1 requires xRXR, and both cognate ligands.** Transcriptional activity was monitored with the TRE-reporting vector, pSEAP (TRE). (a) Lanes 1 and 2 are negative (pSEAP) and positive (pSEAP) vector controls. Oocytes expressing TRA1 or TRA1 plus xRXR, were incubated with 100 nM T3 (lanes 3–5) plus 100 nM RA (lane 5) for 3 d. Cytosolic extracts from each group of oocytes were 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 is present only in oocytes expressing TRA1 and xRXR, exposed to both T3 and RA. (b) Transcriptional activity of TRA1 requires the pBox within the DBD and the NLS. Oocytes expressing xTR, xRXR, and xTR1/xRXR, show no SEAP immunoreactivity when incubated with T3 (lane 6) or T3 plus RA (lane 7). Western blot analysis shows that xRXR, TRA1, and xTR1/xRXR are expressed at comparable levels (Western blots below lanes 4–7). TRA1 and xTR1/xRXR were detected with the monoclonal mouse anti-human TRs antibody (MA1-215). xRXR, was detected with a polyclonal rabbit anti-human RXR antibody (Sc-774).

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**Figure 3. Acute modulation of Ca2+ signaling does not require heterodimerization of TRA1 with xRXR.** (a) Spatial-temporal stacks of IP3-induced Ca2+ wave activity in control oocytes compared with oocytes expressing TRA1 or TRA1/xRXR, T3 (100 nM) and RA (100 nM) were added as indicated 10–15 min before injection with IP3 (~300 nM). Scale is the same as Fig. 1. (b) Western blots of oocytes expressing TRA1 and xRXR. 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.
xRXRα were initially exposed to both T3 (100 nM) and RA (100 nM) 10 min before injection with IP3 (~300 nM). The average Ca2⁺ interwave period for xTRβA1-expressing oocytes was 6.58 ± 0.26 s (n = 67), whereas that of xRXRα/xTRβA1 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 Ca2⁺ 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 xRXRα coexpression does not affect the ability of T3-bound xTRβA1 to modulate Ca2⁺ signaling.

The DNA binding domain and NLS of TRβA1 are not required for acute affects on Ca2⁺ signaling

The ability of T3-bound xTRβA1 to rapidly modulate Ca2⁺ 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 Ca2⁺ signaling. Oocytes were injected with the p-TRE-SEAP plasmid reporting vector. This reporter system requires heterodimerization of xRXRα and xTRβA1 to transactivate the reporter gene (Fig. 2 a). Consequently, test oocytes were co.injected with xRXR mRNA with mRNA encoding either wild-type xTRβA1 (control), mutant xTRβA1 lacking the NLS (xTRβA1ΔNLS), or the mutant lacking both the NLS and the pBOX (xTRβA1ΔpBox-NLS). Once injected, oocytes were continuously bathed in T3 (100 nM) and RA (100 nM) for 3 d. Expression levels of xTRβA1 mutants and xRXRα groups were comparable to xTRβA1 and xRXRα groups (Fig. 2 b, bottom). Using the expression of SEAP as a marker for transcriptional activity, we confirmed that oocytes expressing the xTRβA1 mutants and xRXRα proteins were transcriptionally inactive, whereas oocytes expressing wild-type xTRβA1 and xRXRα proteins exhibited strong transcriptional activity (Fig. 2, lanes 7 and 5).

Subsequently, we tested whether the transcriptionally inactive xTRβA1 mutants could still acutely regulate Ca2⁺ signaling. Oocytes were injected with xTRβA1 mRNA or its mutants and protein expression levels were confirmed using Western analysis 2–3 d after injection (Fig. 4 c). Oocytes expressing xTRβA1 or the mutants were exposed to T3 (100 nM) 10 min before injection with IP3 (~300 nM). Ca2⁺ activity was confocally imaged, as described above. The average Ca2⁺ 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 xTRβA1 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 Ca2⁺ wave period in oocytes expressing either the single mutant xTRβA1ΔNLS (9.6 ± 0.48 s, n = 24) or the double mutant, xTRβA1ΔpBox-NLS (8.4 ± 0.28 s, n = 24) was indistinguishable from oocytes expressing wild-type xTRβA1 (Fig. 4, b and d). We conclude from these data that neither the pBOX nor the NLS of TRβA1 is required for acute regulation of Ca2⁺ signaling.

T3-bound TRβA1 appears to regulate Ca2⁺ signaling by increasing mitochondrial respiration

We reported previously that pyruvate/malate-energized mitochondria increase the amplitude and interwave period of IP3-induced Ca2⁺ waves in Xenopus oocytes (Jouaville et al., 1995). These effects on Ca2⁺ wave activity were similar to those observed in TRβA1 overexpressing oocytes with acute T3 incubation (Fig. 1). Sterling and colleagues (Sterling, 1980) initially reported that T3 increases mitochondrial metabolism, particularly oxidative phosphorylation, in less than 30 min. Consequently, we hypothesized that the regulation of Ca2⁺ signaling by T3-activated xTRβA1 was mediated by its acute modulation of mitochondrial metabolism, which, in turn, increased mitochondrial Ca2⁺ uptake. Our strategy to test this hypothesis was threefold. First, we examined the effect of T3 on ΔΨ in TRβA1 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-
injected a subgroup of the TR\textsubscript{β}A1 expressing oocytes with ru-thenium 360 (Ru\textsubscript{360}; Calbiochem, \textcopyright1 \mu M final concentration), a polycation that inhibits the electrogenic mitochondrial Ca\textsuperscript{2+} uniporter (Ying et al., 1991) \textasciitilde60 min before IP\textsubscript{3} injection and Ca\textsuperscript{2+} imaging. A control group of TR\textsubscript{β}A1-expressing oocytes with buffer only. We found that Ru\textsubscript{360} treatment completely inhibited the effect of T\textsubscript{3}-bound TR\textsubscript{β}A1 on Ca\textsuperscript{2+} wave activity (Fig. 6). Untreated TR\textsubscript{β}A1 expressing oocytes exhibited the expected increase in wave periodicity (7.65 \pm 0.4 s, \( n = 11 \)) when preexposed to T\textsubscript{3} for 10 min. However, the average wave period of Ru\textsubscript{360}-treated TR\textsubscript{β}A1 expressing oocytes was only 5.75 \pm 0.22 s (\( n = 13 \)) when preexposed to T\textsubscript{3}. The Ru\textsubscript{360}-treated average was nearly identical to untreated control oocytes (5.59 \pm 0.04 s, \( n = 4 \)) as well as Ru\textsubscript{360}-treated nonexpressing oocytes (5.53 \pm 0.4 s, \( n = 3 \)). These data are consistent with the hypothesis that T\textsubscript{3}-bound TR\textsubscript{β}A1 regulates Ca\textsuperscript{2+} signaling by increasing mitochondrial Ca\textsuperscript{2+} uptake via an increase in \( \Delta \Psi \).

Third, we directly test whether thyroid hormone receptor together with T\textsubscript{3} stimulates mitochondrial respiration. Xenopus oocytes were injected with TR\textsubscript{β}A1 mRNA or water and incubated for 3 d. The rate of O\textsubscript{2} consumption was measured as an indicator of respiration. 200 oocytes in each group were loaded into a 2-ml O\textsubscript{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\textsubscript{2} consumption was monitored for 30 min. The medium was exchanged a third time with MBS containing 100 nM T\textsubscript{3} and O\textsubscript{2} consumption was followed for another 30 min (Fig. 7, a and b). After this protocol, the rate of O\textsubscript{2} consumption in water-injected oocytes after T\textsubscript{3} exposure was 0.42 \pm 0.25 nmol/min (\( n = 8 \)). In contrast, the rate of O\textsubscript{2} consumption in TR\textsubscript{β}A1-injected oocytes after T\textsubscript{3} exposure was significantly increased to 1.68 \pm 0.52 nmol/min (\( n = 4 \), \( P < 0.05 \)). These data support the hypothesis that a T\textsubscript{3} / TR\textsubscript{β}A1-mediated increase in mitochondrial respiration was responsible for the modulation of IP\textsubscript{3}-mediated Ca\textsuperscript{2+} wave activity.

**TRs targeted to the mitochondria are required for a T\textsubscript{3}-stimulated increase in respiration and the regulation of Ca\textsuperscript{2+} signaling**

T\textsubscript{3} treatment has previously been reported to increase mitochondrial metabolism (Sterling et al., 1980; Soboll, 1993a).
Our data suggest that the acute effects of T3 on mitochondrial metabolism are likely to be mediated by T3-activated thyroid hormone receptors. A truncated form of rat TR1 (rTR1ΔF) has been shown to localize to mitochondria matrix (Ardail et al., 1993; Wrutniak et al., 1995; Casas et al., 1999). Furthermore, the NH2-terminus of the Xenopus TRaA1 that we used throughout this work has a high homology to the NH2-terminus of rTR1ΔF (Fig. 8 a). Our strategy in this experiment was to test whether mitochondrial targeting of TRs was necessary to modulate Ca2+ signaling. First, we examined the cellular targeting of xTRaA1, rTR1, and rTR1ΔF 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 xTRaA1 and rTR1ΔF were detected in the mitochondria extracts. Full-length rTR1 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 O2 consumption for oocytes expressing either rTRa1 or rTRa1ΔF (Fig. 9). Consistent with its mitochondrial targeting, the rate of O2 consumption in rTRa1ΔF expressing oocytes after T3 exposure was significantly increased 1.88 ± 0.35 nmol/min (n = 3, P < 0.05). In contrast, the rate of O2 consumption in oocytes expressing the full-length rTRa1 was not significantly affected by T3 exposure (−0.16 ± 0.55, n = 3). We conclude that mitochondrial targeting of TRs is required for a T3-mediated increase in mitochondrial respiration.

Finally, we tested whether targeting of TRs to mitochondria was required to regulate Ca2+ signaling. As before, oocytes were injected with either full-length rTRa1 or NH2 terminus–truncated rTRa1ΔF mRNAs. Protein expression levels were measured 2–3 d after injection (Fig. 10 b). Ca2+ activity was confocally imaged 10 min after treatment with T3 (100 nM). We found that the average Ca2+ interwave period for rTRa1ΔF–injected oocytes was 8.8 ± 0.26 s (n = 24), which was significantly higher (ANOVA single factor, P < 0.01) than full-length rTRa1–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 Ca2+ signaling by T3-activated TRs requires their localization within mitochondria.
Discussion

In this work, we report that the acute exposure of oocytes expressing mitochondrially targeted TR to T3 regulates IP3-mediated Ca2+ wave activity. We observed a T3-bound TR induced increase in the Ca2+ wave period and amplitude. These changes in Ca2+ 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 IP3-mediated Ca2+ release was due to an increase in mitochondrial Ca2+ uptake via an increase in the ΔΨ. Our current work is consistent with this model because we could inhibit the effects of T3-bound TRs by inhibiting mitochondrial Ca2+ uptake with Ru360. We also directly demonstrated that T3 exposure increased ΔΨ in oocytes expressing TRs. An increase in ΔΨ 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 T3 to mitochondria has been reported to decrease proton leak in several preparations (Crespo-Armas and Mowbray, 1987; Soboll, 1993a). However, we found that T3 exposure increased O2 consumption in TR-expressing oocytes. An increase in the rate of O2 consumption is not consistent with a decrease in proton leak. Together, our data favors the conclusion that T3-bound TR regulates Ca2+ activity by increasing ΔΨ 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 T3 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 T3 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 Mg2+ from mitochondria. On the other hand, Wrutniak and coworkers (Wrutniak-Cabello et al., 2001) found no evidence demonstrating a direct interaction between ANT and T3. 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 TR1 (rTR1ΔF) 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 ΔA1, which is highly similar to rTR1ΔF, targeted the mitochondria. Casas and coworkers (Casas et al., 1999) reported that mitochondrial activity was stimulated by overexpression of p43 (mitochondria-targeted, truncated-TRs), 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 T3 as the full-length TRs 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 NH2 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 Ca2+ wave activity with the same efficacy as the wild type, xTR ΔA1. We confirmed that xRXR Δ was required for xTR ΔA1 to transactivate a reporter gene in our system, but more importantly, the presence of xRXR did not affect the ability of xTR ΔA1 to modulate Ca2+ activity. Thus, we concluded that the mechanism by which T3-activated TRs regulate Ca2+ 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-bounded 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 T3 on mitochondria. Rather, they demonstrate that classic TRs, those that have long been known to regulate gene transcription, but not on its ability to initiate transcription. Together, these observations reveal a nontranscriptional targeting of the TR, but not on its ability to initiate transcription.

Evans, The Howard Hughes Medical Institute, Chevy Chase, MD, and The Rhoten, 1995), and thyroid hormone (Hummerich and Soboll, 1989), and vitamin D3 (Sergeev and 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-bounded 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 T3 on mitochondria. Rather, they demonstrate that classic TRs, those that have long been known to regulate gene transcription, but not on its ability to initiate transcription. Together, these observations reveal a nontranscriptional targeting of the TR, but not on its ability to initiate transcription.

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hancer for the SEAP gene (Merckury Pathway Profiling SEAP System2; CLONTECH, Laboratories, Inc.). The negative control vector pSEAP m-RE 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 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 1, rTR 1, xTR 1, rTR 1, ΔΔ) were allowed to express for 4 d and then treated with 100 nM T 3 for 15 min at RT. Oocytes were washed twice times with buffer A (in mM: 195 sorbitol, 1 CaCl 2, 10 EDTA, pH 7.4) and resuspended in buffer A at a final volume of 500 μl. Oocytes were sequentially homogenized with a handheld homogenizer and centrifuged at 1,0000 × 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 TE5, pH 7.4) and spun at 1,0000 × 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.

O2 consumption assay
A biological O2 monitor (model 5300; YSI Inc.) was used to measure O2 consumption. 200 oocytes in each group were loaded into 2 ml O2 probe chamber avoiding contact of the oocytes with the O2 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 O2 consumption was monitored for 30 min. The media was exchanged again with MBS containing 100 nM T 3 and O2 consumption was followed for the next 30 min. The slope of O2 levels was calculated before and after the addition of T 3.

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