3,5,3'-Tri-iodo-L-thyronine acutely regulates a protein kinase C-sensitive, Ca^{2+} -independent, branch of the hepatic α_1 -adrenoreceptor signalling pathway

Francisco J. DAZA, Roberto PARRILLA and Angeles MARTÍN-REQUERO¹

Department of Pathophysiology and Human Molecular Genetics, Centro de Investigaciones Biológicas (CSIC), Velázquez 144, Madrid-28006, Spain

This work aimed to investigate the acute effect of the thyroid hormone 3,5,3'-tri-iodo-L-thyronine (T_3) in regulating the hepatic metabolism either directly or by controlling the responsiveness to Ca^{2+} -mobilizing agonists. We did not detect any acute metabolic effect of T_3 either in perfused liver or in isolated liver cells. However, T_3 exerted a powerful inhibitory effect on the α_1 -adrenoreceptor-mediated responses. The promptness of this T_3 effect rules out that it was the result of rate changes in gene(s) transcription. T_3 inhibited the α_1 -adrenoreceptor-mediated sustained stimulation of respiration and release of Ca^{2+} and H^+ , but not the glycogenolytic or gluconeogenic responses, in perfused liver. In isolated liver cells, T_3 enhanced the α_1 -agonist-induced increase in cytosolic free Ca^{2+} and impeded the intracellular alkalinization. Since T_3 also prevented the α_1 -adrenoreceptor-mediated activation of protein kinase C, its effects on pH seem to be the result of a lack of activation of the Na^+/H^+ exchanger.

The failure of T_3 to prevent the α_1 -adrenergic stimulation of gluconeogenesis despite the inhibition of protein kinase C activation indicates that the elevation of cytosolic free Ca^{2+} is a sufficient signal to elicit that response. T_3 also impaired some of the angiotensin-II-mediated responses, but did not alter the effects of PMA on hepatic metabolism, indicating, therefore, that some postreceptor event is the target for T_3 actions. The differential effect of T_3 in enhancing the α_1 -adrenoreceptor-mediated increase in cytosolic free Ca^{2+} and preventing the activation of protein kinase C, provides a unique tool for further investigating the role of each branch of the signalling pathway in controlling the hepatic functions. Moreover, the low effective concentrations of T_3 (≤ 10 nM) in perturbing the α_1 -adrenoreceptor-mediated response suggests its physiological significance.

INTRODUCTION

Most metabolic pathways are known to be susceptible to regulation by thyroid hormones. The effect of 3,5,3'-tri-iodo-L-thyronine (T_3) in controlling metabolism can be exerted either directly or through the modulation of nutritional or hormonal signals (reviewed in [1]). Recent work has demonstrated that the T_3 receptors are nuclear proteins that belong to a large superfamily of ligand-activated transcription factors [2]. Thyroid-hormone receptors (TRs) bind to specific DNA sequences named thyroid hormone response elements (TRE) either as homodimers or by forming heterodimers with nuclear receptors for retinoic acid (RAR), retinoid (RXR) or cholecalciferol (vitamin D_3) (ER) [3]. Thus, the current view is that T_3 controls cellular functions through its ability to regulate the transcription of responsive genes [4]. This seems to be the mechanism underlying numerous observations in which the T_3 effects were detected only several hours after its administration, as it is the case for metabolic changes due to the rate changes in the expression of genes encoding key regulatory enzymes.

Short-term, protein-synthesis-independent, effects of T_3 have also been reported [5–7], indicating the existence of mechanism(s) of T_3 action other than transcriptional regulation. The evidence in favour of the existence of extra nuclear receptors for T_3 supports this postulate [8–10]. In this sense, Ca^{2+} homeostasis is known to be perturbed acutely by T_3 . For instance, T_3 produces a transient increase in cytosolic free Ca^{2+} in rat thymocytes [11] and hepatic mitochondria from thyroidectomized rats accumulate Ca^{2+} upon addition of T_3 [12]. It has also been reported that T_3 induces Ca^{2+} uptake and stimulation of respiration within

minutes of the onset of its administration in perfused rat liver [13].

Calcium serves an important function as intracellular messenger. The steady-state level of cytosolic free Ca^{2+} in liver cells is maintained in the range of 0.1–0.2 μM [14–16] by the coordinated action of a number of calcium transport systems in the cellular membranes. The state of activation of α_1 -adrenergic or vasoactive peptides (vasopressin or angiotensin II) receptors influence calcium homeostasis by controlling the intracellular Ca^{2+} mobilization and Ca^{2+} fluxes across the plasma membrane [17,18].

The influence of thyroid status on the adrenergic activation of metabolism has been long recognized [19,20]. From a clinical point of view, thyrotoxicosis resembles increased adrenergic activity [21]. Moreover, the adrenergic blockers are very effective in ameliorating the symptoms of hyperthyroidism. Conversely, in hypothyroidism, changes in the opposite direction to those shown in hyperthyroidism are observed that have been attributed to reduced adrenergic responsiveness. There are numerous examples in the literature that illustrate the putative changes in adrenergic responsiveness induced by thyroid hormones. In rat liver, conflicting results have been obtained, and most studies focused on the β -adrenergic receptors [22]. Despite the important role played by the α_1 -adrenoreceptors in controlling the hepatic metabolism, as far as we know, the acute effect of thyroid hormones on the hepatic α_1 -adrenoreceptor-mediated actions has not been investigated previously.

The present work aimed to investigate whether T_3 was capable of modulating the α_1 -adrenoreceptor-mediated activation of hepatic metabolism. We observed that T_3 acutely inhibits several of the α_1 -adrenoreceptor-mediated effects in rat liver. The in-

Abbreviations used: T_3 , 3,5,3'-triiodo-L-thyronine; TR, thyroid-hormone receptors; TRE, thyroid-hormone response elements; BCECF-AM, 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein tetraacetoxymethyl ester; EIPA, 5-(N-ethyl-N-isopropyl)amiloride; PKC, protein kinase C.

¹ To whom correspondence should be addressed.

Figure 1 Effect of T_3 on α_1 -adrenergic responses of livers from fed rats

Livers isolated from fed rats were perfused in a non-recirculating system with a Krebs–Ringer hydrogen bicarbonate buffer, pH 7.4, until a steady rate of oxygen consumption was attained, then T_3 and phenylephrine (PE) were added sequentially for the periods of time indicated by arrows and concentrations given in the boxes. Data for glucose production are mean $\pm$ S.E. of four–six experiments. The averages $\pm$ S.E. of the sustained changes after PE addition in the absence or in the presence of T_3 were, respectively: oxygen consumption 287 ± 19 and 209 ± 28 $\mu\text{mol/h}$ per 100 g body wt; portal pressure: 2.4 ± 0.1 and 1.5 ± 0.016 mmHg; pH: -0.069 ± 0.008 and -0.045 ± 0.004 unit and $[\text{Ca}^{2+}] = 0.025 \pm 0.002$ and -0.023 ± 0.005 mM. Recordings from representative experiments are shown.

teraction of T_3 with the hepatic α_1 -adrenoreceptor signalling pathway offers the interest of its branch selectivity as far as it potentiates the receptor-mediated increase in the concentration of cytosolic free Ca^{2+} , though the activation of protein kinase C (PKC) is prevented. Thus, according to our results, T_3 becomes a novel and selective tool to elucidate the α_1 -adrenoreceptor-mediated hepatic effects dependent on either the Ca^{2+} or the PKC signalling pathways. Moreover, very low concentrations of T_3 (≤ 10 nM) were sufficient to perturb the hepatic α_1 -adrenergic responsiveness, suggesting its physiological significance.

EXPERIMENTAL PROCEDURES

Animals

Male Wistar rats (body weight 180–220 g) were maintained, until surgery, under controlled lighting and temperature conditions with free access to tap water and a standard laboratory chow diet

(B&Q Universal Limited, Barcelona, Spain) that contained nitrogen-free extract (54.7%), crude protein (16.0%), crude oil (3.0%), fibre (4.5%), ash (6.8%), calcium (1.2%) and phosphorus (0.7%). All the animals were treated in a manner that complied with the European guidelines for the care and use of laboratory animals.

Liver perfusion and isolation of liver cells

Livers from fed or starved rats were perfused with Krebs–Ringer hydrogencarbonate buffer in a flow-through system. Technical details were similar to those previously described [23]. Routinely, the perfusion flow rate was adjusted to 30 ml/min. A 30 min equilibration period was allowed routinely before the infusion of substrates. Substrates and phenylephrine were administered diluted in buffer. Oxygen consumption was determined by measuring the $p\text{O}_2$ arteriovenous difference with a Clark-type

Figure 2 Effect of T_3 on basal and α_1 -adrenoreceptor-induced hepatic responses in starved rats

Livers from 48 h-starved rats were allowed to equilibrate for 30 min until steady rates of oxygen consumption were attained and then T_3 and phenylephrine (PE) were added sequentially for the periods of time indicated by arrows and concentrations given in the boxes. Representative plots are shown. Data for glucose production are means $\pm$ S.E. of, at least, five experiments. The averages $\pm$ S.E. of the sustained changes after 1 μ M PE addition in the absence and in the presence of 100 nM T_3 were, respectively: oxygen consumption: 275 ± 18 and 166 ± 29 μ mol/h per 100 g body wt.; portal pressure: 2.3 ± 0.5 and 1.54 ± 0.2 mmHg; pH: -0.020 ± 0.01 and 0.024 ± 0.01 unit and $[Ca^{2+}] = 0.031 \pm 0.001$ and -0.02 ± 0.005 mM. Data shown for the effects of 10 μ M PE in the presence of 10 nM T_3 are the mean of two experiments.

oxygen electrode.. The pH, pK^+ and pCa^{2+} were continually monitored in the outflow perfusate, using selective electrodes. The portal-vein pressure was measured with a pressure transducer, model Hugo Sach Elektronik (March-Hugstetten, Germany). The amplified analog signals from the different sensors were digitized in a Hewlett-Packard analog-digital converter model HP 3421A, and the data imported into a desk computer through a IEEE-488 communication port for further processing. Liver cells were obtained by perfusion of the hepatic vascular system with collagenase according to previously described protocols [24].

Measurement of cytosolic Ca^{2+} and intracellular pH

Intracellular $[Ca^{2+}]$ and pH were determined using the intracellular trappable fluorescent Ca^{2+} and pH indicators fura 2 and

2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF), respectively, [25,26].

Incubation, loading of the hepatocytes with fura2 acetoxy-methyl ester or BCECF-AM and the measurements of agonist-mediated changes in cytosolic free Ca^{2+} levels and pH_i were performed as described previously [24,27]. To measure Ca^{2+} inflow, liver cells were depleted of internal Ca^{2+} by incubation in nominally Ca^{2+} -free medium in the presence of the endomembranes Ca^{2+} -ATPase inhibitor, thapsigargin [28]. The rate of Ca^{2+} inflow was estimated as the increase in $[Ca^{2+}]_i$ observed after restoring the normal extracellular levels of Ca^{2+} .

Fluorescence measurements were carried out using a Perkin-Elmer LS-50 B spectrofluorimeter equipped with a fast filter accessory that minimizes the interval delay between changing wavelengths. Calibrations of the fluorescent signals were carried out using either the method of Grynkiewicz et al. for Ca^{2+} [29] or the nigericin/ K^+ method of Thomas et al. for pH [30].

Protein kinase C activity

Isolated hepatocytes (100 mg wet wt/ml) were incubated in Krebs–Ringer hydrogencarbonate buffer, pH 7.4, containing 2% Ficoll-70 for 5 min in the absence or in the presence of 100 nM T_3 , followed by the addition of 10 μ M phenylephrine, 10 nM angiotensin II or 100 nM PMA for an additional period of 15 min. Cytosolic and solubilized plasma-membrane fractions were obtained as previously described [24]. PKC activity was assayed in both fractions by measuring the incorporation of 32 P from [γ - 32 P]ATP into histone (type III-SS, Sigma), essentially as described in [31]. The assay mixture (200 μ l) contained 1 mM Tris, pH 7.5, 7.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 50 μ g histone, 25 μ g phosphatidylserine, 1 μ g 1,2-dioctanoylglycerol, 0.05 mM ATP, 0.25 μ Ci [γ - 32 P]ATP and 50 μ l of the enzyme preparation. Basal activity was obtained by replacing $CaCl_2$ and phospholipids with 5 mM EGTA. The mixture was incubated at room temperature for 10 min. The reaction was stopped by the addition of 2 ml of 5% trichloroacetic acid containing 10 mM H_3PO_4 and 200 μ g of bovine serum albumin. The precipitates formed were collected and washed on Whatman GF/A filters and the radioactivity counted in a liquid-scintillation counter.

Metabolite analysis

Perfusate samples were analysed immediately after their collection and assayed spectrophotometrically, using previously described enzymic procedures [32].

Materials

All reagents were of the highest purity available, most were obtained from Sigma (St. Louis, MO, U.S.A.). The enzymes and BCECF were obtained from Boehringer, Mannheim. [γ - 32 P]ATP (5000 Ci/mmol) was obtained from Amersham (Amersham, Bucks., U.K.). EIPA [5-(*N*-ethyl-*N*-isopropyl)amiloride] was obtained from Research Biochemicals International (Natick, MA, U.S.A.).

Statistics

Comparisons between means were carried out by two-tailed unpaired Student's *t* test or, when appropriate, analysis of variance followed by the Newman–Keuls multiple comparison test. Differences were considered to be significant at $P < 0.05$.

RESULTS

Effect of T_3 on the α_1 -adrenoreceptor-mediated stimulation of hepatic metabolism

Figure 1 (left-hand traces) depicts the hepatic responses to the α -adrenergic agonist phenylephrine in perfused livers of rats fed *ad libitum*. In agreement with previous reports [23,33], phenylephrine stimulated oxygen uptake, glucose mobilization, H^+ and Ca^{2+} release and increased vascular resistance. The continuous infusion of T_3 did not alter any of these parameters; however, its presence produced remarkable effects on the hepatic responses to α_1 -adrenergic stimulation (Figure 1, right-hand traces). The stimulation of respiration and extracellular acidification were inhibited, a net uptake of Ca^{2+} was observed, and the glycogenolytic response did not seem to be significantly affected. Whether T_3 was administered before or combined with the α_1 -adrenergic responses was better appreciated in livers from starved rats (Figure 2) in which the glycogen stores are virtually depleted and the extracellular release of H^+ due to its co-transport with lactate is avoided [23,24]. In this condition, T_3 inhibited the α_1 -

Figure 3 Effect of T_3 on α_1 -adrenergic-induced responses of livers from starved rats

Livers from 48 h-starved rats were allowed to equilibrate for 30 min until steady rates of oxygen consumption were attained and then T_3 and phenylephrine (PE) were added sequentially for the periods of time and concentrations indicated in the boxes. The experiment was repeated three times, obtaining similar results in each case; a representative experiment is presented.

adrenergic stimulation of all the parameters recorded except glucose release (Figure 2); a net uptake of Ca^{2+} and extracellular alkalization instead of acidification are now clearly observed in T_3 -treated livers (Figure 2, middle traces). Increasing, by two orders of magnitude the ratio of α_1 -agonist to T_3 (10 nM T_3 and 10 μ M phenylephrine) produced a mild effect only on the T_3 inhibition of the α_1 -adrenergic-induced increase in portal pressure (Figure 2, right-hand traces).

Administration of T_3 to perfused livers from 48 h-starved rats, after the onset of the α_1 -agonist administration, produced an almost immediate and progressive inhibition of the α_1 -adrenoreceptor-mediated effects (Figure 3). The action of T_3 persisted when the infusion of T_3 was discontinued (Figure 3).

To determine the effect of T_3 on the α_1 -adrenergic-induced stimulation of gluconeogenesis, we utilized livers from starved rats perfused with near-physiological concentrations of pyruvate. As shown in Figure 4, T_3 did not alter either the basal rates of oxygen consumption and glucose production from pyruvate or the α_1 -adrenergic-induced stimulation of gluconeogenesis.

Effect on T_3 on the angiotensin-II-mediated stimulation of hepatic functions

To investigate if the T_3 action in decreasing the hepatic responsiveness to α_1 -adrenoreceptor activity was a receptor-specific event, we studied the effect of T_3 on the hepatic responses to the Ca^{2+} -mobilizing hormone angiotensin II. Unlike the α_1 -adrenergic agonists, angiotensin II does not produce sustained stimulatory effects on respiration, extracellular acidification, vascular

Figure 4 Effect of T_3 on α_1 -adrenergic-mediated stimulation of gluconeogenesis

Livers from 48 h-starved rats were allowed to equilibrate for 30 min until steady rates of oxygen consumption were attained and then 1 mM pyruvate was added to the perfusion fluid. After 20 min of perfusion with pyruvate, 1 mM pyruvate, phenylephrine (PE) (1 μ M) was infused into the medium for the period of time indicated in the boxes. Results of glucose production are means for four experiments.

smooth muscle contraction or Ca^{2+} release (Figure 5, left hand traces). In contrast with the α_1 -agonist, angiotensin II produced a net uptake of Ca^{2+} while it was infused (Figure 5, left panel). It appears that T_3 decreases the angiotensin-II-induced increase in oxygen consumption, Ca^{2+} uptake and portal pressure (Figure 5, right-hand traces). The presence of T_3 leads to an extracellular alkalization in response to angiotensin II (Figure 5 right hand trace). No effect of T_3 on the angiotensin-II-induced glucose release was observed (Figure 5, lower traces).

Effects of T_3 on agonist-induced Ca^{2+} mobilization

As expected, the α_1 -agonist phenylephrine and the vasoactive peptide angiotensin II caused a rapid increase in $[Ca^{2+}]_i$ in isolated hepatocytes (Figure 6, a and b). This effect was enhanced when liver cells were exposed to T_3 before receptor activation (Figure 6, c and d). In contrast with a previous report [13], we failed to detect any effect of T_3 on the basal intracellular Ca^{2+} levels.

To determine whether T_3 affected selectively the intracellular mobilization of Ca^{2+} or its influx from the extracellular medium, we investigated its effect in perturbing the α_1 -adrenergic or angiotensin-II-induced increase in cytosolic free Ca^{2+} in hepatocytes maintained in nominally Ca^{2+} -free medium. In the absence of extracellular Ca^{2+} , the plateau phase of the response to Ca^{2+} mobilizing agonists was markedly reduced (Figure 6, traces e and f). In this condition, T_3 did not alter the acute and transient increase in cytosolic free Ca^{2+} induced by either α_1 -agonists or angiotensin II (Figure 6, traces g and h), ruling out an effect of T_3 on intracellular Ca^{2+} mobilization. This point was further investigated using cells in which the intracellular inositol 1,4,5-trisphosphate-sensitive Ca^{2+} pool had been depleted by incubation in Ca^{2+} -free medium containing 0.5 μ M thapsigargin and subse-

quently challenged by restoring the normal extracellular Ca^{2+} level, in the absence or in the presence of phenylephrine, with and without T_3 . Data in Figure 7 show that α_1 -adrenergic stimulation slightly enhanced the rate of increase in cytosolic free $[Ca^{2+}]$ upon re-addition of external Ca^{2+} and this effect was increased by up to 75 % in the presence of T_3 . These results suggest that the effect of T_3 in enhancing the agonist-evoked Ca^{2+} -mobilizing receptor-mediated increase in cytosolic free Ca^{2+} is the result of rate changes in the influx of extracellular Ca^{2+} .

Effect of T_3 on agonist-induced intracellular alkalization

We have recently reported that activation of hepatic α_1 -adrenoceptors is accompanied by activation of the Na^+/H^+ exchanger, resulting in extracellular acidification and intracellular alkalization [23,24,27]. In agreement with these reports, Figure 8 shows that activation of α_1 -adrenergic or angiotensin II receptors produced intracellular alkalization in isolated hepatocytes (Figure 8, left-hand traces). Pretreatment of cells with T_3 for 5 min markedly reduced the agonist-induced intracellular alkalization (Figure 8, right hand traces). Non-receptor-mediated activation of PKC by PMA produced an intracellular alkalosis similar to that induced by the Ca^{2+} -mobilizing agonists (Figure 8). However, non-receptor-mediated activation of PKC was T_3 -insensitive (Figure 8). This fact is consistent with the observation that T_3 did not impair the actions of PMA in isolated perfused liver (results not shown). These observations suggest that thyroid hormone acts on one or more step(s) between the Ca^{2+} -mobilizing receptors and PKC activation. We have further investigated this point by studying the effect of T_3 on the agonist-mediated activation of PKC. In agreement with previous observations [33,34], either the α_1 -adrenoreceptor stimulation or the treatment with PMA translocates the cytosolic PKC towards the plasma

Figure 5 Effect of T_3 on the angiotensin-II-mediated responses of livers from starved rats

Livers from 48 h-starved rats were allowed to equilibrate for 30 min until steady rates of oxygen consumption were attained in the absence and in the presence of 100 nM T_3 , and then 100 nM angiotensin II (ANG II) was added for the period of time indicated by the boxes. Values shown for glucose output are the mean of, at least, three experiments. Representative traces of oxygen consumption, extracellular pH and Ca^{2+} are shown.

membrane in liver cells (Figure 9). Previous exposure of liver cells to T_3 significantly impaired the α_1 -agonist or angiotensin-II-mediated translocation and stimulation of PKC (Figure 9). In contrast, T_3 was ineffective in the non-receptor-mediated activation of PKC induced by PMA (Figure 9).

DISCUSSION

Interaction of T_3 with the hepatic α -adrenoreceptor signalling pathway

Unlike previous work in which T_3 was shown to induce Ca^{2+} uptake and stimulation of respiration within minutes of the onset of its administration [13], we failed to detect any direct hepatic effect of T_3 . The reason for this discrepancy is not clear at present, but it could be due to differences in the concentration of T_3 utilized or in the nutritional or hormonal status of the rats. The possibility should also be considered that as yet undetermined factors present in the perfusate could interact with T_3 . However, T_3 exhibited a powerful acute effect in modulating the hepatic responsiveness to Ca^{2+} -mobilizing agonists, particularly the α_1 -agonists (Figure 1, 2 and 4). This observation is of particular interest considering that concentrations of T_3 within the physiological levels [35] were effective in perturbing the α_1 -adrenergic responses. It is known from previous studies that T_3 might

Figure 6 Effect of T_3 on α_1 -adrenergic and angiotensin-II-mediated increase in cytosolic free $[Ca^{2+}]$ in isolated hepatocytes

Loading of hepatocytes with fura 2 and measurement of cytosolic Ca^{2+} were performed as described in the Experimental procedures section. Each trace is representative of observations from, at least, four different cell preparations. In traces a, b, c and d, the hepatocytes were incubated in buffer containing 1.3 mM $CaCl_2$ in the absence or in the presence of 100 nM T_3 for 5 min. When indicated by the arrows, 10 μ M phenylephrine (PE) or 10 nM angiotensin II (ANG II) was added. In traces e, f, g and h, cells were incubated in nominally Ca^{2+} -free medium. Basal $[Ca^{2+}]_i$ was 225 ± 21 nM and was not changed by T_3 .

perturb Ca^{2+} homeostasis. For instance, T_3 produces a transient increase in Ca^{2+} inflow and/or cytosolic free Ca^{2+} concentration in rat myocardium [36] or in rat thymocytes [11], as well as accumulation of Ca^{2+} in hepatic mitochondria from thyroidectomized rats [12]. However, most of these observations have been made using supraphysiological concentrations of T_3 and, as far as we know, no acute, direct, interaction of T_3 with receptor-mediated processes has ever been reported.

The thyroid status alters the number and/or affinity of the hepatic α_1 -adrenoreceptors [20,22,37]. Nevertheless, the promptness of the T_3 effect in perturbing the hepatic α_1 -adrenergic responsiveness rules out that it could act by modulating the transcription of responsive genes. However, the following observations suggest that T_3 did not alter the α_1 -adrenoreceptor ligand-binding affinity: (i) T_3 impaired neither the α_1 -agonist-mediated mobilization of intracellular Ca^{2+} , nor the glycogenolytic or gluconeogenic responses; (ii) T_3 also perturbed some of the angiotensin II receptor-mediated responses (Figure 5); and finally (iii) a 100-fold increase in the α_1 -agonist/ T_3 ratio did not alter the T_3 effects on the hepatic α_1 -adrenergic responses. Thus it seems plausible to conclude that some postreceptor event must be the target for the T_3 action. This possibility agrees with the postulate of the involvement of sites of T_3 action other than those leading to changes in number or affinity of adrenergic receptors to explain the influence of the thyroid status on the hepatic β -adrenergic responsiveness [38].

We have previously shown that hepatic α_1 -adrenergic-induced sustained release of Ca^{2+} and H^+ was the result of activation of the Na^+/H^+ antiporter and the functional coupling of the Na^+/H^+

Figure 7 Effect of T_3 on α_1 -adrenergic-mediated stimulation of Ca^{2+} influx in isolated hepatocytes

Loading of cells with fura 2 and fluorescence measurements were performed in nominally Ca^{2+} -free medium. Depletion of internal Ca^{2+} was accomplished as described in the Experimental procedures section in the presence of $0.5 \mu M$ thapsigargin. When indicated by the arrow, $1.3 mM$ $CaCl_2$ was added. Traces shown are representative of at least five observations carried out in cells incubated in the absence or in the presence of $10 \mu M$ phenylephrine (PE) with and without $100 nM$ T_3 . The average maximal changes in cytosolic $[Ca^{2+}]_i \pm S.E.$ are presented in the lower panel. Differences between mean values after addition of phenylephrine with and without T_3 were found to be statistically significant from basal values ($P < 0.05$).

and Na^+/Ca^{2+} exchangers [23]. The latter effects were controlled by a PKC-dependent α_1 -adrenergic signalling pathway [27]. In contrast, glucose mobilization and gluconeogenesis relied on the operation of a Ca^{2+} -mobilization-dependent signalling mechanism. The present investigation adds further evidence in support of the operation of at least two independent α_1 -adrenergic receptor signalling pathways in rat liver. This assertion is based on the differential effects of T_3 on the α_1 -adrenergic-induced Ca^{2+} mobilization and the intra- and extra-cellular pH changes. Parallel effects were shown by T_3 in preventing the α_1 -adrenergic-induced activation of PKC, H^+ release, intracellular alkalization and Ca^{2+} release (Figures 1, 2 and 3), indicating their cause-effect relationship. In addition, T_3 impaired neither the non-receptor-mediated activation of PKC (Figure 9) nor the PMA-induced intracellular alkalosis, an effect that ultimately relies on the activation of PKC [39] (Figure 8). These observations support the conclusion that T_3 prevents the activation of the Na^+/H^+ exchanger as a result of the lack of activation of PKC. It also seems plausible to conclude that T_3 interacts with the α_1 -adrenoreceptor signalling pathway at some postreceptor step(s) before the PKC activation. In contrast with the α_1 -adrenoreceptor-mediated pH changes, T_3 did not perturb the elevation of cytosolic free Ca^{2+} or the glycogenolytic and gluconeogenic responses (Figures 1–4). Thus, a cytosolic Ca^{2+} elevation is a sufficient signal to elicit an α_1 -adrenergic-mediated stimulation of glucose synthesis and mobilization. It also allows one to conclude that a Ca^{2+} -sensitive PKC-independent, α_1 -adrenoreceptor signalling pathway controls glycogenolysis and gluconeogenesis. According to these observations T_3 could be of aid in discerning the association of a given α_1 -adrenergic response to a PKC and/or a Ca^{2+} -mobilization-dependent signalling pathway.

Effect of T_3 on the Ca^{2+} -mobilizing, agonist-induced changes in Ca^{2+} fluxes

The hepatic α_1 -adrenoreceptor or angiotensin II activation elicit a rapid biphasic increase in the cytosolic free Ca^{2+} followed by a

Figure 8 Effect of T_3 on α_1 -adrenergic-angiotensin-II and PMA-mediated stimulation of Na^+/H^+ in isolated hepatocytes

Loading of hepatocytes with BCECF and measurement of intracellular pH were performed as described in the Experimental procedures section. Each trace is representative of observations from at least four different cell preparations. The average maximal changes in intracellular pH induced by these agonists, in the absence or in the presence of $100 nM$ T_3 , were, respectively: 0.032 ± 0.003 unit and 0.012 ± 0.003 unit ($P < 0.01$) after addition of $10 \mu M$ phenylephrine (PE); 0.027 ± 0.002 unit and 0.008 ± 0.001 unit ($P < 0.001$) after addition of $10 nM$ angiotensin II (ANG II); and 0.027 ± 0.004 unit and 0.025 ± 0.004 unit ($P > 0.05$) in the presence of $100 nM$ PMA. Basal intracellular pH was 7.05 ± 0.019 and was not changed by the presence of T_3 .

Figure 9 Effect of T_3 on α_1 -adrenergic-, angiotensin-II- and PMA-mediated stimulation of PKC in isolated hepatocytes

Isolated hepatocytes were incubated in Krebs–Ringer hydrogen carbonate buffer, pH 7.4, containing 2% (w/v) Ficoll-70 for 15 min in the absence or in the presence of 10 μ M phenylephrine (PE) or 10 nM angiotensin II (ANG II). When indicated, cells were preincubated for 5 min in the presence of 100 nM T_3 . PKC was assayed in cytosol and solubilized plasma membrane as described in the Experimental procedures section. Results are expressed as the percentage change of soluble and membrane-associated PKC activity from basal values, which were, respectively, 78 ± 6 and 41 ± 4 pmol/min per mg of protein. Values shown are the means $\pm$ S.E. of triplicate determinations carried out in, at least, five different cell preparations. The observed changes in PKC activity after addition of phenylephrine, angiotensin II and PMA were found to be significantly different from basal values ($P < 0.001$). In the presence of T_3 , no differences are observed after hormone addition ($P > 0.05$) while PMA is still able to activate the enzyme ($P < 0.001$).

period of sustained Ca^{2+} elevation that depends on the continuous presence of both agonists and extracellular calcium [17,18]. The presence of T_3 enhanced the α_1 -agonist- or angiotensin-II-mediated increases in the concentration of cytosolic free Ca^{2+} (Figure 6). The augmented Ca^{2+} signal observed in T_3 -treated hepatocytes (Figure 6) seems to be the consequence of an enhanced plasma membrane Ca^{2+} inflow. This conclusion is supported by the observations that the peak height of the α_1 -adrenergic- or angiotensin-II-induced Ca^{2+} signal was not significantly affected by T_3 in liver cells incubated in nominally calcium-free medium (Figure 6), and that T_3 enhanced the phenylephrine-mediated Ca^{2+} entry (Figure 7). These observations add further support to the postulated role of T_3 in modulating calcium inflow in other types of cells [11,36].

Despite the fact that phenylephrine and angiotensin II induced a similar increase in intracellular Ca^{2+} mobilization and Ca^{2+} entry from the extracellular medium in isolated hepatocytes (Figure 6), they produced opposite changes in Ca^{2+} levels in the outflow perfusate (Figures 1, 2 and 5). The Ca^{2+} levels in the effluent are set by the balance of two opposing forces, the passive influx from the extracellular medium and the active extrusion of Ca^{2+} from the cell against the electrochemical gradient. Therefore,

the effects of phenylephrine and angiotensin II in inducing a net release or uptake of Ca^{2+} , respectively, might indicate a differential action on the rate of Ca^{2+} efflux. Additionally, the possibility should be considered that the α_1 -adrenergic agonist and angiotensin II could affect in a different manner the contribution of non-parenchymal cells to net Ca^{2+} levels in the hepatic outflow. Further work will be needed to clarify these points.

The mechanism of agonist-stimulated extracellular Ca^{2+} entry has not yet been elucidated. The experimental data suggest three possible mechanisms: (1) receptor-activation of Ca^{2+} channels through a G-protein(s) [40]; (2) second-messenger-operated Ca^{2+} channels [41,42], and, finally, (3) a capacitative calcium entry, in which activation of Ca^{2+} entry is linked to emptying of an internal, inositol 1,4,5-trisphosphate-sensitive, Ca^{2+} store [43]. These mechanisms may not be mutually exclusive. The effect of T_3 in enhancing the α_1 -adrenergic-stimulated Ca^{2+} inflow (Figure 7), but not the release of Ca^{2+} from intracellular stores (Figure 6), makes it unlikely that T_3 would alter the formation of inositol phosphates or the size of the internal calcium stores. In addition, T_3 stimulated the vasopressin and angiotensin-II-mediated increase in cytosolic free Ca^{2+} (Figure 6). We have previously demonstrated the involvement of a pertussis-toxin-sensitive G-protein in both α_1 -adrenoreceptor- and vasopressin-mediated hepatic calcium fluxes [44]. Moreover, the angiotensin-II- or vasopressin-stimulated Ca^{2+} inflow in single hepatocytes has been shown to require a slowly ADP-ribosylated pertussis-toxin-sensitive GTP-binding regulatory protein [40,45]. On these grounds, it is tempting to speculate that T_3 might enhance the α_1 -adrenergic-induced Ca^{2+} signal by interacting with the G-protein(s) implicated in the control of the receptor-activated Ca^{2+} inflow system. The interaction of this regulatory G-protein with the Ca^{2+} -inflow transporter seems to be a phospholipase C independent process [45]. Since PKC seems to modulate Ca^{2+} inflow in a number of cell types [46,47], the possibility should be also considered that T_3 impeded the α_1 -agonist-induced stimulation of calcium inflow as a result of the lack of PKC activation.

To conclude, T_3 interacts with the hepatic α_1 -adrenoreceptor signalling system at some postreceptor step(s), beyond the stimulation of phospholipase C, leading to a selective inhibition of PKC translocation to the plasma membrane. Since inositol 1,4,5-trisphosphate and diacylglycerol, the activator of PKC, are produced simultaneously by the action of phospholipase C, an explanation for the T_3 effect in dissociating the α_1 -agonist-induced responses might be that T_3 interacted exclusively with the non-phosphoinositide-linked processes implicated in the sustained generation of diacylglycerol [48,49] without affecting the transient production of diacylglycerol induced by phospholipase C activation. The present observations on the acute effects of T_3 support the existence of at least two independent α_1 -adrenoreceptor signalling pathways: one is PKC-dependent, Ca^{2+} -independent and T_3 sensitive; the second is PKC-independent, Ca^{2+} -mobilization-dependent and T_3 insensitive.

The skilful technical assistance of M. J. Arias-Salgado is greatly appreciated. This work has been supported in part by grants from Fondo de Investigaciones Sanitarias (94/0224), Direccion General de Investigación Científica y Técnica (DGICYT) PB94-1544, and DGICYT 93/163.

REFERENCES

- 1 Oppenheimer, J. H. and Samuels, H. H. (1983) *Molecular Basis of Thyroid Hormone Action*, Academic Press, New York
- 2 Evans, R. M. (1988) *Science* **240**, 889–895
- 3 Yen, P. M. and Chin, W. W. (1994) *Trends Endocrinol. Metab.* **5**, 65–72
- 4 Brent, G. A., Moore, D. D. and Larsen, P. R. (1991) *Annu. Rev. Physiol.* **53**, 17–35
- 5 Adamson, L. F. and Ingbar, S. H. (1967) *Endocrinology* **81**, 1362–1371
- 6 Muller, M. J. and Steitz, H. J. (1980) *Life Sci.* **27**, 827–835

- 7 Chen-Zion, M., Bassukevitz, Y. and Beitner, R. (1995) *Biochem. Mol. Med.* **56**, 19–25
- 8 Kato, H., Fukuda, T., Parkinson, C., McPhie, P. and Cheng, S.-Y. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7861–7865
- 9 Kitagawa, S., Obata, T., Hasumura, S., Pastan, I. and Cheng, S.-Y. (1987) *J. Biol. Chem.* **262**, 3903–3908
- 10 Hagiwara, M., Mamiya, S. and Hidaka, H. (1989) *J. Biol. Chem.* **264**, 40–44
- 11 Segel, J. and Ingbar, S. H. (1984) *Endocrinology* **115**, 160–166
- 12 Herd, P. A. (1978) *Arch. Biochem. Biophys.* **188**, 220–225
- 13 Hummerich, H. and Soboll, S. (1989) *Biochem. J.* **258**, 363–367
- 14 Joseph, S. K. and Williamson, J. R. (1983) *J. Biol. Chem.* **258**, 10425–10432
- 15 Charest, R., Blackmore, P. F., Berthon, B. and Exton, J. H. (1983) *J. Biol. Chem.* **258**, 8769–8773
- 16 Reinhart, P. H., Taylor, W. M. and Bygrave, F. L. (1983) *Biochem. J.* **214**, 405–412
- 17 Putney, Jr., J. W. (1986) *Cell Calcium* **7**, 1–12
- 18 Fasolato, C., Innocenti, B. and Pozzan, T. (1994) *Trends Pharmacol. Sci.* **15**, 77–83
- 19 Menahan, L. A. and Wieland, O. (1969) *Eur. J. Biochem.* **10**, 188–194
- 20 Malbon, C. C. and LoPrelli, J. J. (1981) *J. Biol. Chem.* **256**, 12199–12204
- 21 Irvine, W. J. and Toft, A. D. (1976) *Clin. Endocrinol.* **5**, 687–707
- 22 Bilezikian, J. P. and Loeb, J. N. (1983) *Endocr. Rev.* **4**, 378–388
- 23 Urcelay, E., Butta, N., Ciprés, G., Martín-Requero, A., Ayuso, M. S. and Parrilla, R. (1994) *J. Biol. Chem.* **269**, 860–867
- 24 Ciprés, G., Butta, N., Urcelay, E., Parrilla, R. and Martín-Requero, A. (1995) *Endocrinology* **136**, 468–475
- 25 Kass, G. E. N., Duddy, S. K., Moore, G. A. and Orrenius, S. (1989) *J. Biol. Chem.* **264**, 15192–15196
- 26 Rink, T. J., Tsien, R. Y. and Pozzan, T. (1982) *J. Cell. Biol.* **95**, 189–196
- 27 Butta, N., Martín-Requero, A., Urcelay, E., Parrilla, R. and Ayuso, M. S. (1996) *Br. J. Pharmacol.* **118**, 1797–1805
- 28 Thastrup, O., Cullen, P. J., Dobrak, B. K., Hanley, M. R. and Dawson, A. P. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2466–2470
- 29 Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3340–3450
- 30 Thomas, J. A., Buschbaum, R. N., Zimniak, A. and Racker, E. (1979) *Biochemistry* **18**, 2210–2218
- 31 Kikawa, U., Tokai, Y., Minakuchi, R., Inchara, S. and Nishizuka, Y. (1982) *J. Biol. Chem.* **257**, 13341–13348
- 32 Bermeyer, H. U. (1975) *Methods in Enzymatic Analysis*, Academic Press, New York
- 33 Urcelay, E., Butta, N., Manchón, C. G., Ciprés, G., Requero, A. M., Ayuso, M. S. and Parrilla, R. (1993) *Endocrinology* **133**, 2105–2115
- 34 Hernández-Sotomayor, S. M. T. and García-Saínz, J. A. (1988) *Biochim. Biophys. Acta* **968**, 138–141
- 35 Oppenheimer, J. H. and Schwartz, H. L. (1985) *J. Clin. Invest.* **75**, 147–154
- 36 Götzsche, L. B.-H. (1994) *Eur. J. Endocrinol.* **130**, 171–179
- 37 Preiksaitis, H. G., Kan, W. H. and Kunos, G. (1982) *J. Biol. Chem.* **257**, 4321–4327
- 38 Malbon, C. G. and Greenberg, M. L. (1982) *J. Clin. Invest.* **69**, 414–426
- 39 Ives, H. E. and Daniel, T. O. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1950–1954
- 40 Berven, L., Hughes, B. P. and Barritt, G. J. (1994) *Biochem. J.* **299**, 399–407
- 41 Berridge, M. J. (1993) *Nature (London)* **361**, 315–325
- 42 Montero, M., García-Sancho, J. and Alvarez, J. (1993) *J. Biol. Chem.* **268**, 13055–13061
- 43 Putney, Jr., J. W. (1990) *Cell Calcium* **11**, 611–624
- 44 Butta, N., Urcelay, E., González-Manchón, C., Parrilla, R. and Ayuso, M. S. (1993) *J. Biol. Chem.* **268**, 6081–6089
- 45 Fernando, K. C. and Barritt, G. J. (1994) *Biochem. J.* **303**, 351–356
- 46 McCarthy, S. A., Hallam, T. J. and Merritt, J. E. (1989) *Biochem. J.* **264**, 357–364
- 47 Törnquist, K. (1993) *Biochem. J.* **290**, 443–447
- 48 Penner, R., Matthews, G. and Neher, E. (1987) *Nature (London)* **334**, 499–504
- 49 Exton, J. H. (1990) *J. Biol. Chem.* **261**, 1–4