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Rapid Nongenomic Effects of 3,5,3′-Triiodo-L-Thyronine on the Intracellular pH of L-6 Myoblasts Are Mediated by Intracellular Calcium Mobilization and Kinase Pathways

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L-T_3 and L-T_4 activated the Na^+/H^+ exchanger of L-6 myoblasts, with a fast nongenomic mechanism, both in the steady state and when cells undergo acid loading with ammonium chloride. Monitored with the intracellular pH-sensitive fluorescent probe 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein, activation of the exchanger appeared to be initiated at the plasma membrane, because T_3-agarose reproduced the effect of L-T_3, and triiodothyroacetic acid, a hormone analog previously shown to inhibit membrane actions of thyroid hormone, blocked the action of L-T_3 on the exchanger. We show here for the first time that transduction of the hormone signal in this nongenomic response requires tyrosine kinase-dependent phospholipase C activation and two different signaling pathways: 1) mobilization of intracellular calcium, assessed by the fluorescent probe fura-2, through activation of inositol trisphosphate receptors and without contributions from extracellular calcium or ryanodine receptors; and 2) protein phosphorylation involving protein kinase C and MAPK (ERK1/2), as shown by the use of kinase inhibitors and by immunoblotting for activated kinases. (*Endocrinology* 145: 5694–5703, 2004)

THE NA^+/H^+ EXCHANGER type 1 (NHE-1) is a ubiquitous integral plasma membrane protein that exchanges extracellular Na^+ for cytoplasmic H^+ according to concentration gradients (1–3). It modulates intracellular pH (pH_i) and cell volume. The exchanger does not require energy for activity but depends indirectly on the Na^+/K^+-ATPase. Activity is also influenced by growth factors, mitogens, hormones, integrins, and hyperosmolarity (4–7). NHE-1 is a phosphoglycoprotein of 815 amino acids that contains Ca^{2+}/calmodulin binding sites and several sites of phosphorylation (1, 8). Regulation of the exchanger at the molecular level can occur through at least three different mechanisms: 1) interaction of a regulatory factor with a critical cytoplasmic region of NHE-1; 2) direct phosphorylation of serine, threonine, or tyrosine residues in the cytoplasmic domain, or 3) Ca^{2+}/calmodulin binding (3). Several protein kinases may be involved in the phosphorylation of NHE-1, including Ca^{2+}/calmodulin-dependent kinase (8), protein kinase C (PKC) (8, 9), phosphatidyl inositol 3-kinase (9), and members of the MAPK pathways (2, 10). The rapid activation of NHE-1 by growth factors and hormones is usually associated with phosphorylation of the cytoplasmic domain (1). After deletion of the cytoplasmic tail with its potential phosphorylation sites (amino acids 636–815), however, approximately 50% of the activation achieved upon exposure of cells to growth factors is preserved, suggesting that other pathways are operative for full activation (11).

Nongenomic actions of thyroid hormone have been described at the level of the plasma membrane, cytoskeleton, cytoplasm, and organelles of mammalian cells (12). Some of these actions rapidly lead to posttranslational modification of nucleoproteins, *e.g.* serine phosphorylation of the nuclear thyroid hormone receptor TRβ1 (13), estrogen receptor (14), and p53 (15). These hormone actions on nucleoproteins are mediated by MAPK (ERK1/2). Upstream of MAPK, PKC and the phosphatidylinositol pathway may be activated by iodothyronines (12–16). These effects of the hormone have been demonstrated in cells that lack functional nuclear thyroid hormone receptors (15, 16). The mechanisms by which thyroid hormone nongenomically affects the activity of plasma membrane ion channels and ion pumps (17) are not well understood and are the focus of the present report.

Activation of MAPK by thyroid hormone (L-T_3) and rapid modulation of plasma membrane ion pumps imply the existence of discrete membrane receptors for the hormone that may be linked to signal transduction pathways. Membrane binding sites for thyroid hormones were identified years ago in cell membranes from human and rat erythrocytes (18–21) and rat hepatocytes (22, 23) (for a recent review see Ref. 24). For L-T_3, two stereospecific binding sites were detected in these membranes; one with apparent dissociation constant

Abbreviations: BAPTA/AM, 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethylester); BCECF/AM, 2′,7′-Bis(carboxyethyl)-5(6)-carboxyfluorescein tetra-acetoxymethylester; DAG, diacylglycerol; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; IP_3, inositol trisphosphate; NHE-1, Na^+/H^+ exchanger type 1; $[NH_4^+]_i$, intracellular NH_4^+ concentration; pH_i, intracellular pH; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PTU, 6-*n*-propyl-2-thiouracil; RyR, ryanodine receptor; tetrac, deaminated T_4; triac, 3,3′,5-triiodothyroacetic acid.

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(K_d) values in the lower nanomolar range, and the other with K_d in the (sub)micromolar range. Linkage between such binding sites and hormone actions has not been established. One of our laboratories has recently shown that the extracellular domain of a structural membrane protein, integrin $\alpha V\beta 3$, is capable of binding thyroid hormone and that such binding activates MAPK (25). Occlusion of the site prevents activation of MAPK by iodothyronines and prevents cellular actions of the hormone downstream of MAPK. Thus, a cell surface binding site for the hormone has been identified that is associated with certain nongenomic effects of thyroid hormone.

Thyroid hormone has both genomic (26–29) and nongenomic (30, 31) effects on the activity of the Na^+/H^+ exchanger. Evidence that the *primum movens* in nongenomic effects of thyroid hormone was an increase in intracellular calcium was first shown in 1989 by Segal and Ingbar in rat thymocytes (32). The authors concluded that the increased calcium could be relevant to the activity of adenylate cyclase, cAMP concentration, and 2-deoxyglucose uptake. Warnick *et al.* (33) later showed that skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase activity is thyroid hormone responsive. It has been postulated that the effect of thyroid hormone on the Ca^{2+}-ATPase of sarcoplasmic reticulum may contribute to the shortened relaxation phase found in patients with thyrotoxicosis (34).

Physiological concentrations of thyroid hormone increase pH_i through activation of the Na^+/H^+ exchanger, both at the steady state and after an acid load with ammonium chloride, in cultured L-6 myoblasts from rat skeletal muscle (30). These results also indicate a physiological role for thyroid hormone in the recovery processes after muscle contraction and consequent acidosis. Rapid nongenomic effects of thyroid hormone have been reported also in chick embryo hepatocytes at different stages of embryonic development on two plasma membrane transport systems: the Na^+/H^+ exchanger and the amino acid transport system A (31). Both transport systems were activated through a transduction pathway involving PKC, phosphatidyl inositol 3-kinase, and the MAPK pathway (31).

In the present studies, we have determined whether the reported nongenomic activation by thyroid hormone of the Na^+/H^+ exchanger in L-6 myoblasts is mediated by the same pathways involved in the transduction of nongenomic actions of this hormone in other cell types. In particular, we have examined the potential role of intracellular calcium levels. Our data indicate that at least two major pathways are operative in the nongenomic activation of the NHE-1 by thyroid hormones: intracellular calcium mobilization and kinase activation. Additionally this work confirms that L-T_4 behaves as a prohormone in the L-T_3 action on the Na^+/H^+ exchanger in L-6 cells.

Materials and Methods

Cell culture

L-6 cells from rat skeletal muscle were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 g/ml streptomycin, and 100 U/ml penicillin in an atmosphere of 5% CO_2 at 37 C and were kept in culture as myoblasts by continuous passages at preconfluent stages.

Determination of pH_i

For the fluorescence assays, cells were grown in chamber slides (Lab-Tek, Nunc, Naperville, IL) and used at confluency. Before the experiment, cells were rendered quiescent by serum deprivation for 5 h. Intracellular pH was measured using the pH_i indicator 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein tetra-acetoxymethylester (BCECF/AM). To rule out the contribution of HCO_3^--dependent transport mechanisms (35), all experiments were carried out in bicarbonate-free buffer with the following composition (mM): 135 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 20 HEPES (pH 7.3). This buffer (henceforth called Na^+ buffer) was used for the incubation with the fluorescent probe and for the determination of pH_i unless otherwise stated; the cells incubated in this buffer were considered virtually depleted of bicarbonate.

To carry out experiments with an acid load, solutions containing NH_3/NH_4^+ were prepared from the above buffer with replacement of 20 mM NaCl with 20 mM NH_4Cl, and the remaining external Na^+ was routinely replaced by equimolar choline chloride to keep the antiport quiescent. Incubation with the fluorescent dye was carried out as follows: cells were washed twice with Na^+ buffer and were thus considered bicarbonate free. Cells were then incubated in Na^+ buffer with BCECF/AM at the final concentration of 1 μg/ml for 20 min at 37 C in the dark. Then the medium containing the dye was eliminated, and the cells were washed twice with the Na^+ buffer.

Routinely at the end of each experiment, calibration of fluorescence *vs.* pH was carried out by an established method (36) using the K^+-H^+ ionophore nigericin (5 M) added to cells suspended in a K^+ buffer; the latter had the same composition as the Na^+ buffer but with NaCl replaced by equimolar concentrations of KCl. Under these conditions, intracellular and extracellular pH are equilibrated ($pH_i = pH_o$). The extracellular pH was changed with 10-μl aliquots of 1 M 2-[*N*-morpholino]ethanesulfonic acid or 1 M Tris and measured with a glass electrode inserted directly into the cuvette. Intracellular fluorescence was determined and plotted *vs.* extracellular pH. The calibration curve was linear over the pH range 6.5–7.8 (data not shown).

Fluorescence was measured under continuous magnetic stirring at a controlled temperature (37 C) in a PerkinElmer (Norwalk, CT) LS-50B luminescence spectrometer equipped with a fast filter accessory for the dual-excitation single-emission ratio technique. Excitation wavelengths were set at 498 nm (pH-dependent component) and 450 nm (pH-independent component) with emission at 530 nm, using 5- and 10-nm slits, respectively, for the two light paths. This allowed measurements of pH_i that were not dependent on cell concentration and dye loading (37).

Determination of intrinsic β_i

The total intracellular buffering capacity β_t is defined as: $\beta_t = \beta_{CO2} + \beta_i$. In the nominally HCO_3^--free solutions used in this study, β_{CO2} was assumed to be negligible and β_t was therefore taken to be equal to β_i. The NH_4^+ pulse technique was used to determine β_i, as previously described (38) according to the following formula: $\beta_i = \delta[NH_4^+]_i/\delta pH_i$, where $\delta[NH_4^+]_i$ represents the change in concentration of intracellular NH_4^+ after exposure to or removal of extracellular NH_3, and δpH_i represents the corresponding change in pH_i. The $[NH_4^+]_i$ during the NH_4Cl pulse was calculated as previously reported (38) from the following equation: $[NH_4^+]_i = [NH_3]_i \times 10^{8.92-pH_i}$, taking into account that NH_3 equilibrates across the cell membrane (*i.e.* $[NH_3]_i = [NH_3]_o$) and that the pK_a of NH_4^+ (8.92) is the same intra- and extracellularly. $[NH_4Cl]$ in the absence of added NH_4Cl was assumed to be zero.

From the product of β and the rate of pH_i recovery, the net efflux of acid J can be calculated and expressed as mM/min.

$[Ca^{2+}]_i$ measurement

Cells were used for the determination of $[Ca^{2+}]_i$ from 72–96 h after plating, at 90% confluence. $[Ca^{2+}]_i$ was determined by measuring the fluorescence signal from the Ca^{2+} indicator fura-2, as previously reported (39). The cells in one flask ($\sim 6 \times 10^6$ cells) were loaded with fura-2/AM by incubating them for 30 min in the dark at room temperature in HEPES buffer solution: 20 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5.6 mM glucose, and 1 μM fura-2/AM.

Fura-2/AM is a membrane-permeant Ca^{2+}-insensitive ester of the Ca^{2+} probe fura-2, which becomes Ca^{2+} sensitive and remains trapped

intracellularly after hydrolysis by nonspecific intracellular esterases. After two washes in fura-free HEPES buffer, the cells were scraped and centrifuged for 5 min at 1000 rpm to remove extracellular fura-2/AM. Cells were resuspended in 6 ml HEPES buffer and allowed to recover for 2 h in the same buffer at room temperature to ensure full hydrolysis of the fura-2 ester. The tubes with cells in suspension were wrapped in aluminum foil to protect them from light. For measurements, 600 μl of cell suspension ($\sim 6 \times 10^5$ cells) were dispersed into 3 ml of HEPES buffer.

Fluorescence was recorded at 340- and 380-nm excitation wavelengths and 500-nm emission wavelength (10-nm slit width). All determinations were carried out at 37 C, and full mixing was achieved by magnetic stirring. The fluorescence calibration was initiated by permeabilizing the cells to calcium with the ionophore ionomycin (10 μM) to yield the maximum fluorescence, F_{max}, followed by displacement of calcium from its complex with fura-2 by addition of EGTA (3 mM) to yield F_{min}. The concentration of $[Ca^{2+}]_i$ was reflected in the ratios of the fluorescence intensities of fura-2 emission at 500 nm induced by the alternating excitation wavelengths (340–380 nm) according to the formula (40) $[Ca^{2+}]_i = K_d \times [(F - F_{min})/(F_{max} - F)] \times [(F_{min}\ 380\ nm)/(F_{max}\ 380\ nm)]$, where F, F_{min}, and F_{max} are the fluorescence ratios recorded during the experiment (F) or during calibration tests (F_{min} and F_{max}), and K_d is the fura-2 dissociation constant (150 nM).

Western blot analysis

Subconfluent cells, after hormone treatment, were washed twice in ice-cold PBS and then harvested with trypsin and homogenized with a Teflon pestle into ice-cold 10 mM Tris-HCl (pH 7.4) containing 1 mM $CaCl_2$, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin. Particulate fractions were obtained by centrifuging samples at 100,000 $\times$ *g* for 30 min (41). In other experiments, the harvested cells were sonicated to obtain the total cell lysate (42). Proteins from particulate and total cell lysate were solubilized in 0.125 M Tris-HCl (pH 6.8) containing 10% SDS, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin and boiled for 2 min. Equal amounts (20 μg) of membrane and total proteins were resolved by 7% SDS-PAGE (PKC-α) or 10% SDS-PAGE (ERK and ERK-P) at 100 V for 1 h and then transferred to nitrocellulose filters for 45 min at 100 V and 4 C. The nitrocellulose was treated with 3% BSA in 138 mM NaCl, 26.8 mM KCl, 25 mM Tris-HCl (pH 8.0), 0.05% Tween 20, and 0.1% BSA and then probed at 4 C overnight with anti-ERK-P or anti-PKC-α antibodies (1 μg/ml). The nitrocellulose was stripped by Restore Western blot stripping buffer (Pierce Chemical Co., Rockford, IL) for 10 min at room temperature and then probed with anti-ERK antibody (1 μg/ml). Antiactin antibody (1 μg/ml) was used to normalize the sample loading. Bound antibodies were visualized using enhanced chemiluminescence detection (CDP-Star, NEN Life Science Products, Boston, MA). Protein content was quantified by densitometric analysis of Western blots (Fluor-S, Bio-Rad, Hercules, CA).

Solutions

BCECF/AM (1 mg/ml) and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) (10 mM), genistein, U 73122, H-7, calphostin C, Ro 31-8220, phorbol 12-myristate 13-acetate (PMA), PD 98059, U 0126, geldanamycin, ryanodine, and calmidazolium chloride were dissolved in dimethyl sulfoxide, which did not affect the fluorescence signal at the employed concentrations. Nigericin (10 mM) was dissolved in ethanol. Thyroid hormones and analogs were dissolved initially as 1 mM stock solutions in 0.1 M NaOH and then diluted in buffer to the final desired concentration. 6-*n*-Propyl-2-thiouracil (PTU) (1 mM) and caffeine were aqueous solutions.

Materials

DMEM, antibiotics, and sterile plastic ware for cell culture were from Flow Laboratory (Irvine, UK). Fetal bovine serum was from GIBCO (Grand Island, NY). Fura-2/AM and BCECF/AM were obtained from Molecular Probes (Eugene, OR). Nigericin, ionomycin, HEPES, 2-[*N*-morpholino]ethanesulfonic acid, Tris(hydroxymethyl)aminomethane, L-T_3 (sodium salt), L-T_4 (sodium salt), 3,3′,5-triiodothyroacetic acid (triac), rT_3, PMA, genistein, H-7, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethylester) (BAPTA/AM), EGTA, caffeine, ryanodine, calmidazolium chloride, and activated CH-Sepharose 4B were supplied by Sigma Chemical Co. (St. Louis, MO). D-T_3 (free acid), calphostin C, and PTU were from ICN Pharmaceuticals (Costa Mesa, CA). L-T_3-agarose was prepared as reported in Affinity Chromatography Handbook, Principles and Methods, at www.amershambiosciences.com. EIPA was obtained from Research Biochemicals International (Natick, MA). PD 98059, U 0126, and geldanamycin were from Alexis Biochemicals (Laufelfingen, Switzerland). U 73122 and Ro 31-8220 were obtained from Calbiochem (La Jolla, CA). Monoclonal anti-PKC-α, anti-ERK1/2, anti-ERK1/2-P, and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of the purest grade available from Merck (Darmstadt, Germany).

Results

Effect of thyroid hormone on pH_i

We have previously shown that L-T_3 and L-T_4 activate the Na^+/H^+ antiport in L-6 cells from rat skeletal muscle by a rapid, nongenomic mechanism (30). Representative experiments demonstrating the rapid nongenomic effects of thyroid hormones on the pH_i of L-6 cells are shown in Fig. 1. The effect of L-T_3 (1 nM) on the Na^+/H^+ exchanger was significant when the exchanger activity was measured both at the steady state (Fig. 1A) and under acid load with ammonium chloride (Fig. 1, B and C). Figure 1A also shows the effects of T_3-agarose (1 nM) and L-T_4 (100 nM). When L-6 myoblasts were maintained in a HEPES-buffered solution containing equimolar choline chloride (instead of NaCl) to keep the antiport quiescent and then exposed to NH_4Cl, the pH_i increased rapidly by an average of 0.20 pH units; this was because of diffusion of NH_3. The pH change was followed by a slower return toward the baseline. Abrupt removal of NH_4Cl at this point rapidly decreased the pH_i 0.3–0.4 U because NH_3 left the cell. The readdition of the Na^+ buffer caused a recovery of pH_i; this effect was enhanced when thyroid hormone was added to the cells at the time of recovery, together with the Na^+ buffer (Fig. 1B).

From the regression lines computed from such experiments, it was possible to calculate the rate of recovery in the pH range 6.8–7.2 (Fig. 1C). The addition of L-T_3 at the time of recovery affected both the rate of recovery and the final set-point pH reached by the cells. The x-axis intercepts for control cells, thyroid hormone-treated cells, and thyroid hormone-treated cells plus inhibitor were 7.06, 7.23, and 7.05, respectively. The pH decrease caused by the removal of NH_4^+ was used to evaluate the buffering capacity (β) when the acid transport mechanisms are blocked. The addition of L-T_3 did not significantly affect β, but the rate of recovery and the net efflux of acid J (*i.e.* acid extrusion rate) were significantly increased (Table 1).

To identify the signal transduction pathways involved in the activation of the Na^+/H^+ exchanger by L-T_3 in L-6 myoblasts, we assessed 1) the ability of thyroid hormones to change intracellular calcium ion concentration and 2) the response to thyroid hormone of several signal-transducing protein kinases.

Effect of thyroid hormone on $[Ca^{2+}]_i$

Measurements of $[Ca^{2+}]_i$ with fura-2 showed an abrupt transient peak at approximately 100 sec after addition of L-T_3 (1 nM) (Fig. 2). Also L-T_4 (100 nM) caused an increase in

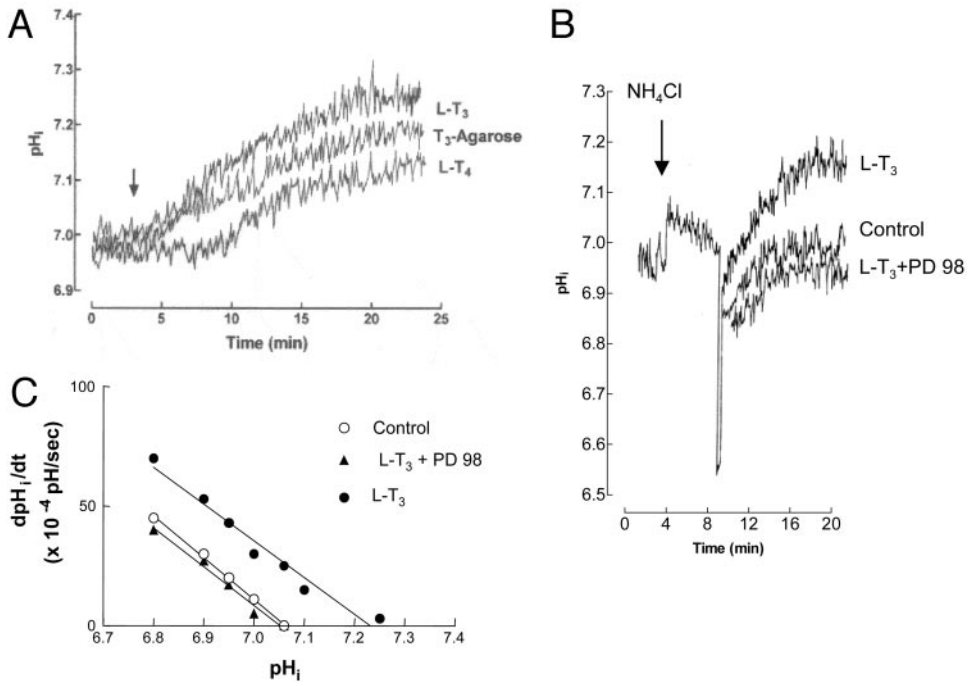

FIG. 1. Effect of L-T_3 on the pH_i at the steady state and after acid load with NH_4Cl in L-6 myoblasts. A, Steady-state measurement of pH_i in the presence of L-T_3 (1 nM), T_3-agarose (1 nM) or L-T_4 (100 nM). The addition of hormones is indicated by the *arrow*. The three thyroid hormone preparations all caused elevation in pH_i over 20 min. B, pH_i recovery from an acid load carried out with a NH_3-NH_4^+ pulse in a choline chloride buffer to keep the exchanger quiescent. Cells were exposed to 20 mM NH_4Cl for 4 min. During the pulse, the pH_i increased (because of entry of NH_3 into the cells) and then decreased toward the baseline because of slower influx of NH_4^+. The abrupt removal of the choline chloride medium containing NH_4Cl and readdition of the Na^+ buffer gave rise to a rapid recovery of pH_i. Also shown is the effect of L-T_3 (10^{-9} M) and hormone plus PD 98059 (10 μM) at the time of recovery. Experiments similar to the one shown were carried out for all inhibitors reported in Table 1, but are not shown. C, Regression lines for control, L-T_3 (1 nM), and L-T_3 plus PD 98059, calculated from recovery experiments similar to those shown in B. The data are the means of four to six experiments.

TABLE 1. Effect of L-T_3 (1 nM) and PKC and MAPK pathway inhibitors on the buffering capacity (β) and acid extrusion rate (J) after acid load experiments with ammonium chloride in L-6 myoblasts

Addition	β	J
None	60 ± 15	16 ± 5
L-T_3	80 ± 24	36 ± 10[a]
L-T_3 and PD 98059	58 ± 13	16 ± 8
L-T_3 and U 0126	55 ± 15	15 ± 5
L-T_3 and geldanmycin	57 ± 12	16 ± 8
L-T_3 and H-7	60 ± 15	16 ± 5
L-T_3 and calphostin C	55 ± 12	18 ± 8
L-T_3 and Ro 31-8220	50 ± 15	16 ± 7

The buffering capacity (β) is reported as mM/pH, whereas the net efflux acid (J), is reported as mM/min. Values are the mean ± SD of three to five different experiments. The values are calculated at pH 6.8. Hormone was given at the time of recovery, whereas the inhibitors, except calphostin C, were given before the acid load as reported in Fig. 1 and in *Materials and Methods*. For calphostin C treatment of cells, see Fig. 5.

[a] $P < 0.05$ at least, with respect to control.

$[Ca^{2+}]_i$ but with a lag time of approximately 6 min (Fig. 2). The difference in the lag times for L-T_3 and L-T_4 was similar to those found in the pH experiments previously reported (30). The effect of L-T_4 on the Ca^{2+} concentration was blocked in the presence of the 5′-deiodinase inhibitor PTU (30), confirming that L-T_4 here behaves as a prohormone leading to L-T_3 formation (data not shown). Calcium mobilization increased with increasing hormone concentration, reflecting the response of the Na^+/H^+ exchanger, whereas the analogs D-T_4, triac, and rT_3, each tested at two selected concentrations, did not show any significant effect (Table 2).

The Ca^{2+} signals had a rather unusual form, being extremely sharp and short-lived. They were very different in shape from the increase of pH that was continuous, reaching a maximum in approximately 20 min. When 10 μM phenylephrine, a well-characterized Ca^{2+} activator, was used, similar signal changes in $[Ca^{2+}]_i$ were obtained (data not shown). It is possible that the efficient Ca^{2+} sequestering system present in the muscle cells could explain this behavior, as found for similar cell types (43). The short lag time indicated that the calcium response was because of a nongenomic effect of L-T_3, most likely mediated through the activation of a cell membrane receptor. This was confirmed in experiments using T_3-agarose, a membrane-impermeant L-T_3 analog, which produced changes in the $[Ca^{2+}]_i$ with exactly the same time course as L-T_3 itself. However, the lag times observed in the studies of pH_i were identical with the ones measured for the calcium response: approximately 100 sec for L-T_3 and T_3-agarose, and approximately 5–6 min for L-T_4, at a concentration 100 times higher (30).

An increase in $[Ca^{2+}]_i$ can be caused by either entrance of extracellular calcium or release of calcium from intracellular stores, or both. The experiments shown in Fig. 3 are in agreement with the hypothesis that intracellular Ca^{2+} mobilization occurs after the addition of L-T_3 to the cells, because the

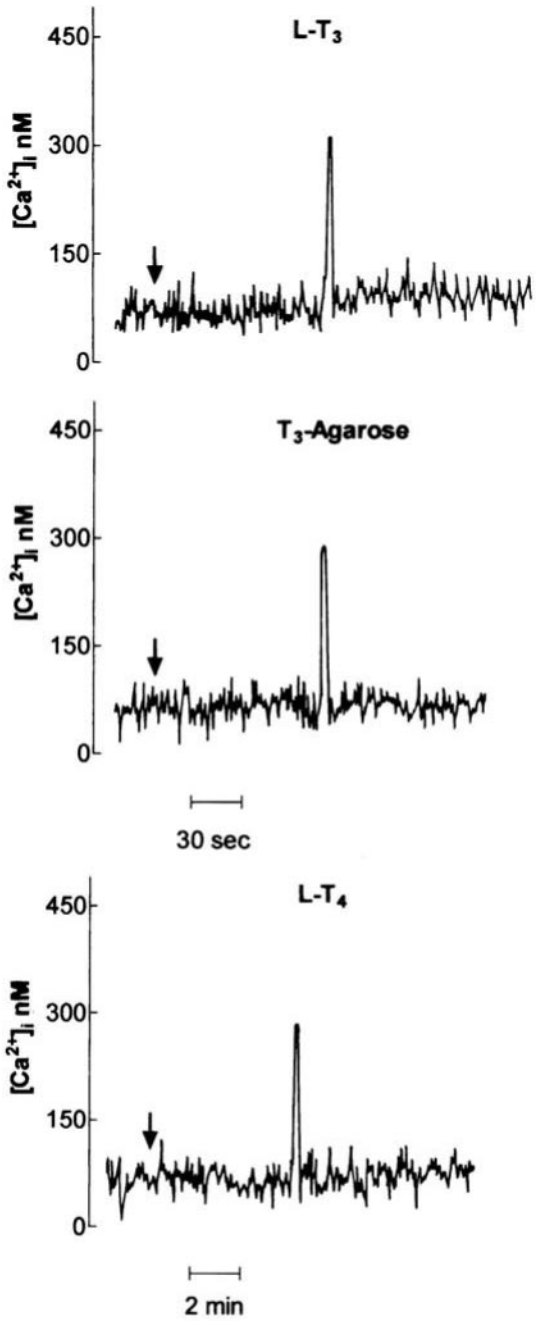

FIG. 2. Effect of thyroid hormones L-T_3, T_3-agarose, and L-T_4 on the $[Ca^{2+}]_i$ in L-6 myoblasts, measured with the fluorescent probe fura-2. L-T_3 and T_3-agarose (1 nM) and L-T_4 (100 nM) were added to cell suspensions (shown by the *arrow*) as described in *Materials and Methods*. Typical curves are shown, representative of at least five to 10 experiments giving very similar traces. The response to L-T_4 was slower than that seen with L-T_3, as indicated by the different time scales; the time scale for L-T_3 and T_3-agarose is the same.

use of Ca^{2+}-free extracellular buffer or the presence of EGTA in the extracellular buffer (data not shown) did not alter the response of L-T_3. In contrast, BAPTA, a selective chelator of intracellular Ca^{2+} stores (44), and U 73122, an aminosteroid phospholipase C (PLC) inhibitor (45), significantly inhibited the activation by L-T_3 (Fig. 3). Triac was minimally effective

TABLE 2. Effect of thyroid hormones and analogs on the intracellular calcium mobilization in L-6 myoblasts

Hormone/analog	1 nM	100 nM
None	70 ± 20	70 ± 20
L-T_3	315 ± 50	450 ± 70[a]
L-T_4	180 ± 45	260 ± 40[b]
D-T_4	75 ± 15	92 ± 30
rT_3	90 ± 20	104 ± 27
Triac	65 ± 15	105 ± 25

Results are given as nM Ca^{2+}, reported as the maximum value of the transient peak, and are the mean ± SD of at least three different experiments.

[a] $P < 0.001$, derived from a Student's t test with respect to L-T_3, 1 nM.

[b] $P < 0.005$ with respect to L-T_4, 1 nM.

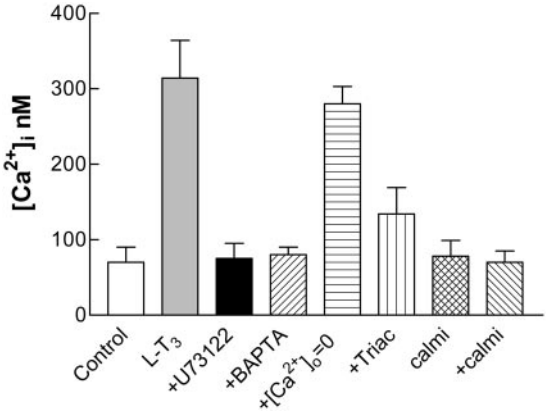

FIG. 3. Effect of different inhibitors, analogs, and chelators on the mobilization of calcium from intracellular stores in L-6 myoblasts. The concentrations used were 1 nM L-T_3, 0.1 μM U 73122, 25 μM BAPTA/AM, 1 μM calmidazolium chloride (calmi), and 1 μM triac. The inhibitors were added to the cells 5 min before hormone addition and did not affect the fluorescent signal. BAPTA/AM, a hydrophobic form of BAPTA that enters the cells and becomes deesterified to BAPTA within, was given to the cells for 30 min. BAPTA alone is not shown for the sake of simplicity, but it did not affect intracellular calcium mobilization. The label +drug stands for L-T_3 plus drug or L-T_3 plus buffer without calcium. Results are reported as concentration of intracellular calcium (nM) and are the mean ± SD of three to 10 different experiments.

by itself (Table 2) but when given together with L-T_3 strongly inhibited the hormone effect on $[Ca^{2+}]_i$ (Fig. 3). Calmidazolium chloride (calmi, 1 μM), an inhibitor of calmodulin, completely prevented the increase in $[Ca^{2+}]_i$ caused by the hormone (Fig. 3).

To characterize further the effect of thyroid hormone on the $[Ca^{2+}]_i$, we used caffeine and ryanodine to assess the possible involvement of ryanodine receptors (46, 47). Both caffeine (5 mM) and ryanodine (100 nM) gave rise to an increase of $[Ca^{2+}]_i$ approximately 30 sec after drug addition (Fig. 4, A and C). When thyroid hormone was given together with either caffeine or ryanodine, the characteristic calcium spike caused by L-T_3 was observed superimposed on the increase caused by either activator (Fig. 4, B and D). These results suggest that the nongenomic effect of L-T_3 was independent of ryanodine receptor activation. The effects of ryanodine and caffeine were evident also in the presence of the inhibitor U 73122 or in a Ca^{2+}-free buffer or with the calcium chelator EGTA in the extracellular medium (data not shown).

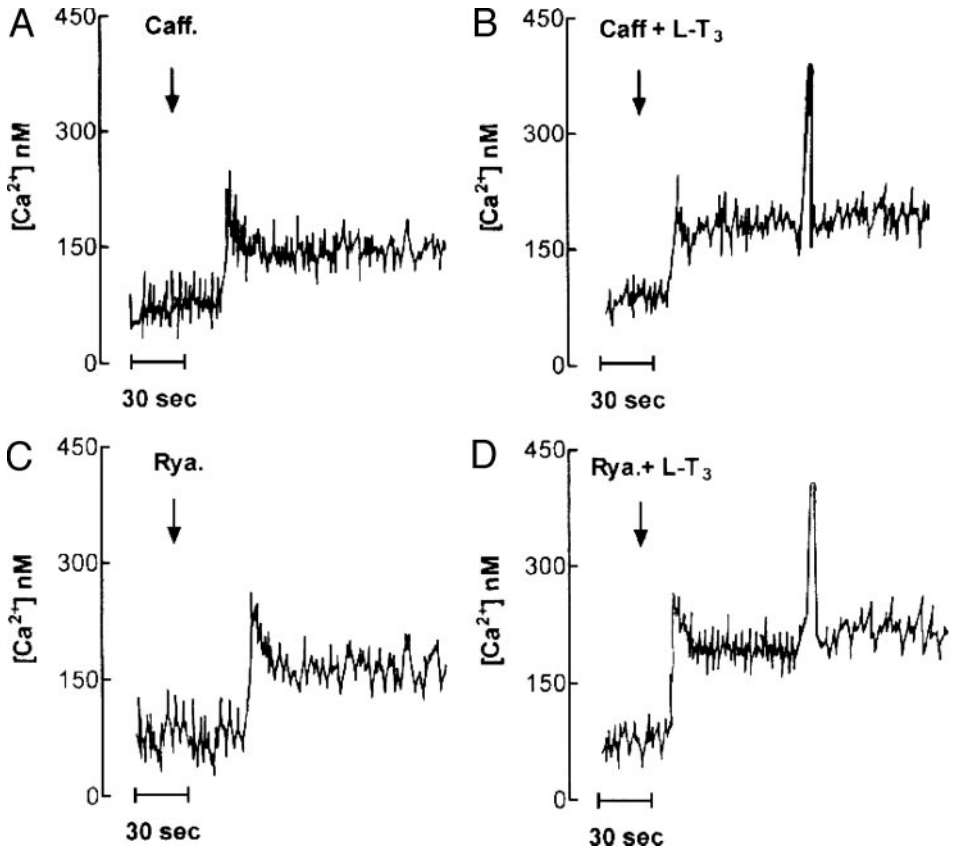

FIG. 4. Effect of caffeine and ryanodine on intracellular calcium mobilization in the presence and absence of thyroid hormone in L-6 myoblasts. Caffeine (5 mM) and ryanodine (100 nM) were assayed alone (A and C) or together with 1 nM L-T_3 (B and D). The *arrow* indicates the addition of either caffeine/ryanodine alone or together with the hormone. The traces reported are representative of at least five different experiments and show a hormone-induced sharp transient increase in $[Ca^{2+}]_i$ in the presence of both agents.

Role of kinases and ion transport inhibitors

The effect of L-T_3 on pH_i was completely blocked by the specific antiporter inhibitor EIPA (10 μM; Fig. 5A). The hormone was not significantly affected by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (0.1 mM), an inhibitor of the Na^+-dependent HCO_3^- transport, indicating that the contribution of this transport system to the increase of pH_i was negligible. Figure 5A also shows that genistein, an inhibitor of tyrosine kinases, almost completely prevented the pH increase caused by hormone. Triac produced a small stimulating effect by itself but when added with L-T_3 completely prevented hormone action (Fig. 5A), analogous to the results of Ca^{2+} experiments with L-T_3 and triac shown in Fig. 3.

We assessed the involvement of two major protein kinase systems, the PKC and MAPK pathways, in the transduction of the nongenomic L-T_3 signal into an effect on pH_i. The PKC inhibitors H-7, calphostin C, Ro 31-8220, and PMA (depletion of PKC activity after overnight incubation) as well as the MAPK pathway inhibitors PD 98059, U 0126, and geldanamycin (48) all prevented the activation of the Na^+/H^+ exchanger by L-T_3, both in the steady state and under acid loading (Fig. 1, B and C; Fig. 5B; and Table 1). Figure 1B shows results of an acid load experiment with ammonium chloride, carried out in the presence or absence of L-T_3, with or without PD 98059, which indicate that the hormone effect is significantly reduced in the presence of the MAPK activation inhibitor. Other common inhibitors of the PKC and MAPK pathways were used in acid load experiments, and their effects on the buffering capacity (β) and acid extrusion rate (J) are reported in Table 1. These results suggest a complex transduction mechanism involving both PKC and the MAPK pathway. None of the inhibitors, when applied alone in the absence of hormone, affected the buffering capacity (β) or the net proton efflux (J). In fact, both parameters depend on the activation of the pH_i sensor, which is not affected by kinase inhibitors (6). Results of inhibitor studies similar to those shown in Fig. 1, B and C, and Table 1 were obtained with L-T_4 (10^{-7} M), using preincubation times longer than 10 min (data not shown).

The effects of L-T_3 on the translocation of PKC-α from the cytosol to the plasma membrane and on activation (phosphorylation) of MAPK (ERK1/2) are shown in Fig. 6. Physiological concentrations of L-T_3 stimulated both PKC translocation and ERK1/2 phosphorylation (Fig. 6, A and C), but these effects disappeared at higher hormone concentrations, in contrast to results obtained in the studies of intracellular calcium mobilization (Table 2). U 73122 inhibited the translocation of PKC-α induced by thyroid hormone, consistent with the inhibition of receptor-induced PLC activation at the cell surface (Fig. 6B). The translocation of PKC was completely inhibited by Ro 31-8220 and by genistein, whereas the specific inhibitor of ERK activation PD 98059 did not affect PKC translocation (Fig. 7A). With regard to the MAPK pathway, we found that the hormone effect on ERK1/2 activation was completely inhibited by genistein, PD 98059 and Ro 31-8220 (Fig. 7B). Thus, activation of the MAPK pathway by thyroid hormone is downstream of activation of PKC.

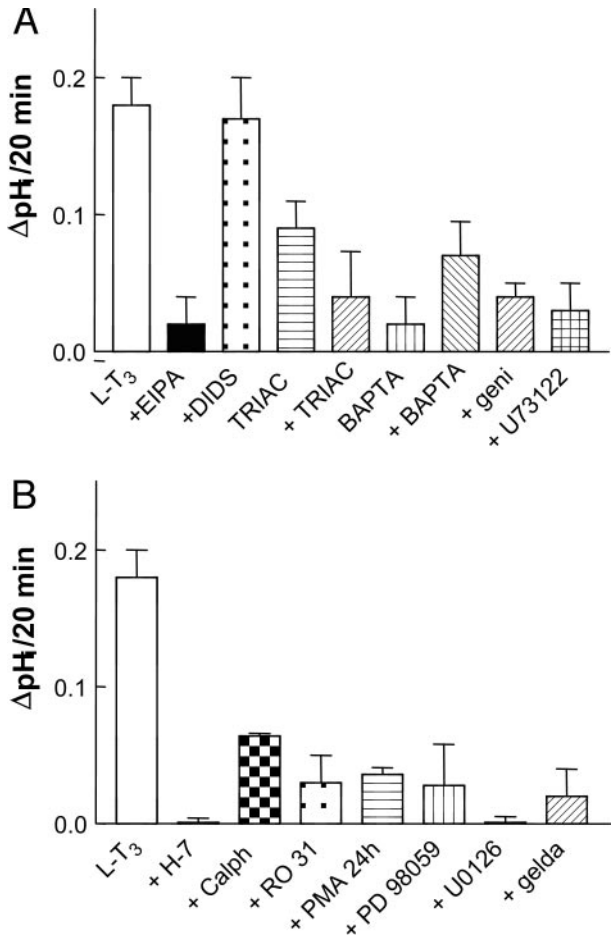

FIG. 5. Effect of the analog triac, inhibitors of transport systems, and inhibitors of tyrosine kinase, PKC, and ERK1/2 activation on the increase of pH_i by L-T_3 at the steady state in L-6 myoblasts. The concentrations of these compounds were 10 μM EIPA, 0.1 mM 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid, 10^{-7} M triac, 25 μM BAPTA, 10^{-7} M genistein (geni), 70 nM U 0126, and 10^{-4} M H-7. Concentrations of U 73122 and light-activated calphostin C (calph) were 1 μM, and Ro 31-8220 (Ro 31) and PD 98059 concentrations were 10 μM. All inhibitors, except calphostin C and PMA, were applied to the cells 5 min before hormone addition. The samples containing calphostin C were light activated with 30 min of dark and 30 min of light before hormone treatment. The samples assayed for down-regulation of PKC with PMA (1 μM) were incubated overnight. The label +drug stands for L-T_3 plus drug. Results are reported as ΔpH_i/20 min over basal value and are mean ± SD of at least three different experiments.

Discussion

Our results show that the fast nongenomic activation by L-T_3 of the Na^+/H^+ exchanger in rat skeletal muscle L-6 cells is mediated in part by an increase in $[Ca^{2+}]_i$ that is independent of a contribution from extracellular calcium. The lag time for the initiation of the response is approximately 100 sec for the effects of hormone on both pH_i and $[Ca^{2+}]_i$, but the time course of the two events is different. The slow increase in pH_i reaches a plateau in approximately 20 min, but there is a spike-type response in calcium. The responses to L-T_4 showed a lag time in onset of 7–10 min, apparently reflecting (PTU inhibitable) deiodination of L-T_4 to L-T_3 for responses both in pH_i and Ca^{2+}.

The nongenomic effect of L-T_3 on pH_i is hormone specific. rT_3, triac, and D-T_4 had negligible effects, individually, on pH_i (30) and on cytoplasmic calcium concentration. Triac added with L-T_3 to L-6 cells, however, inhibited the effects of L-T_3. This result is not surprising, because one of our laboratories has reported that deaminated T_4 (tetrac) can block nongenomic actions of L-T_4 in cells devoid of functional nuclear thyroid hormone receptor (16) as well as in cells that contain TRβ1 (14). We attribute such effects of triac and tetrac to the ability of these deaminated thyroid hormone analogs to compete with thyroid hormone for cell surface hormone-binding sites (13, 16, 49). Although plasma membrane binding sites for iodothyronines have been described in the past for human and rat erythrocytes (18–21) and rat hepatocytes (22–24), it has not been clear that these sites are linked to intracellular events and thus function as receptors. One of our laboratories has recently shown that human integrin $\alpha V\beta 3$ binds thyroid hormone dissociably and that tetrac inhibits association of L-T_4 with this integrin (25). This integrin is a structural plasma membrane protein that is linked to the MAPK signal transduction pathway. Occlusion of the receptor site on the integrin prevents thyroid hormone from activating MAPK and has been shown to inhibit cellular responses to thyroid hormone that are downstream of MAPK. We have proposed that integrin $\alpha V\beta 3$ is the cell surface receptor primarily involved in thyroid hormone's activation of MAPK.

The Na^+/H^+ exchanger can be activated by several mechanisms: interaction of a regulatory factor with a critical cytoplasmic region of NHE-1, phosphorylation at different residues on the cytoplasmic domain, and binding by NHE-1 of the Ca^{2+}/calmodulin complex. A high-affinity calmodulin-binding domain regulates NHE-1 activity in response to Ca^{2+}-dependent signaling mechanisms. At rest, this site may function as an autoinhibitory domain and inhibit ion transport, but upon activation NHE-1 undergoes a conformational change that suppresses the autoinhibitory effect and allows the Ca^{2+}-dependent binding of calmodulin. In the current paper, we show for the first time that the nongenomic effect of thyroid hormone on the Na^+/H^+ exchanger in L-6 myoblasts is mediated by intracellular calcium mobilization. No contribution from extracellular calcium is involved, as shown by the experiments with extracellular and intracellular calcium chelators (EGTA and BAPTA, respectively). BAPTA by itself had no effect on pH_i, but BAPTA plus L-T_3 showed a significant inhibition with respect to hormone alone even though BAPTA did not completely prevent the increase of pH_i, suggesting that other mechanisms that are not dependent on Ca^{2+} contribute to the activation of NHE-1 (*i.e.* phosphorylation by kinases).

We examined two families of calcium channels as possible intracellular sources of the rise in $[Ca^{2+}]_i$ obtained with thyroid hormone: inositol trisphosphate (IP_3) receptors and ryanodine receptors (RyRs) (46, 47). The IP_3 receptor is modulated by the second messenger IP_3 in response to different activators, such as hormones and growth factors, whereas the RyRs are calcium channels that control intracellular calcium levels by releasing calcium from the sarco/endoplasmic reticulum, an intracellular calcium storage compart-

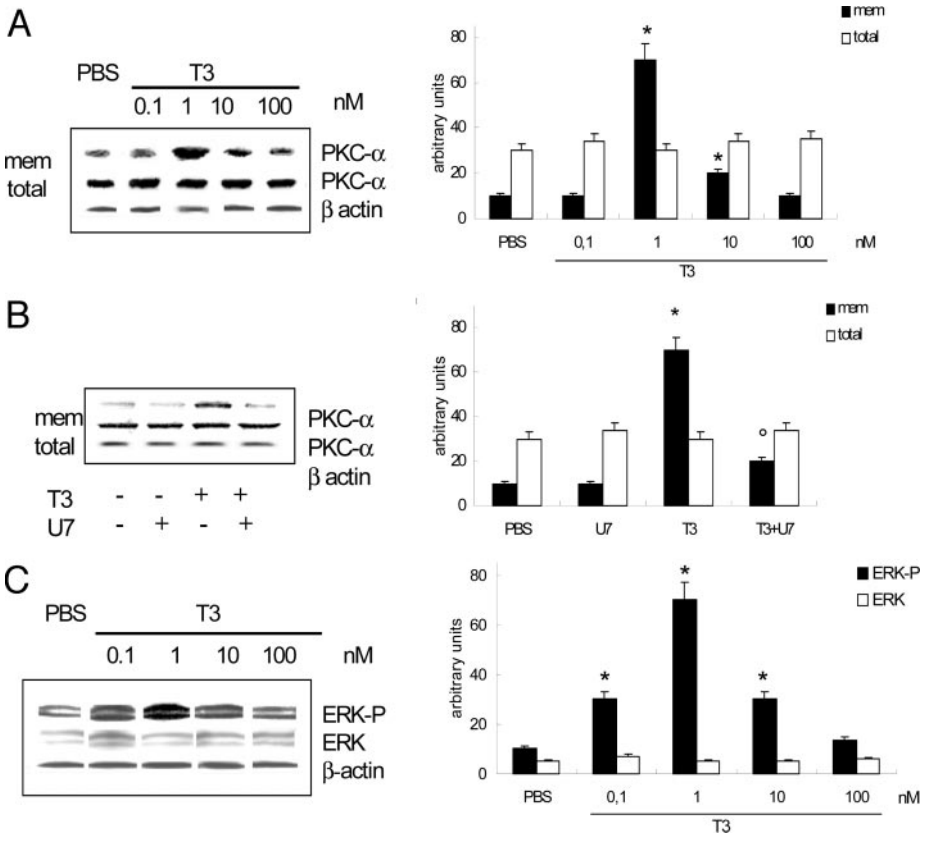

FIG. 6. Effect of L-T_3 on PKC-α translocation and on phosphorylation and content of ERK1/2 and effects of signal transduction inhibitors in L-6 myoblasts. A, Effect of L-T_3, 0.1–100 nM, on translocation of PKC-α from cytosol to the plasma membrane. Membrane-associated PKC-α was significantly increased with L-T_3, 1 nM for 15 min, whereas total cellular PKC-α was unchanged. Immunoblots of β-actin in this and subsequent figures indicate equal sample loading. The graph on the *right* summarizes results of densitometric scanning of immunoblots, as mean ± SD of three to10 similar experiments. B, Effect of the PLC inhibitor U 73122 (1 μM) on the translocation of PKC from cytosol to plasma membrane after treatment with L-T_3 (1 nM). C, Effect of L-T_3 on the content of total ERK1/2 and phosphorylated ERK1/2 (ERK1/2-P) determined by immunoblot. Cells were treated with L-T_3 (1 nM) for 15 min. A maximal amount of ERK1/2-P was evident with 1 nM L-T_3, whereas total ERK1/2 did not change. $P <$ 0.001 as calculated by the Student's t test, with respect to unstimulated (*) or L-T_3-stimulated (°) samples.

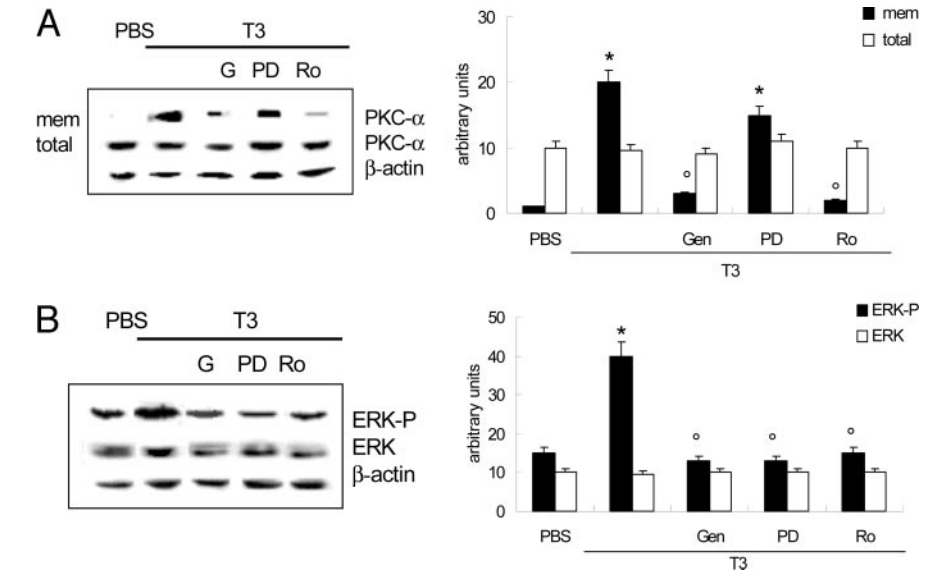

FIG. 7. Effect of selected inhibitors of PKC and the MAPK pathway on translocation of PKC-α to the plasma membrane and the phosphorylation of ERK1/2. The concentrations of inhibitors were the same as reported in Fig. 5. A, Western blots of total and plasma membrane PKC-α after treatment of L-6 cells with L-T_3 (1 nM) and the effect of the inhibitors genistein (G, 0.1 μM), PD 98059 (PD, 10 μM), and Ro 31-8220 (Ro, 10 μM) on the hormone effect. The graph on the *right* corresponds to results of densitometric scanning of immunoblots; the data represent the mean ± SD of three different experiments. B, Parallel studies of ERK1/2 phosphorylation in L-6 myoblasts treated with L-T_3 as in A. The graph on the *right* corresponds to results of densitometric scanning of immunoblots; the data represent the mean ± SD of three different experiments. The β-actin was used to verify the iso-loading of the samples. $P < 0.001$ as calculated by the Student's t test, with respect to unstimulated (*) or L-T_3-stimulated (°) samples.

ment. The RyR channel can be opened by nanomolar concentrations of ryanodine, a plant alkaloid, and by millimolar caffeine. Our data show that L-T_3 and caffeine/ryanodine mobilize different pools of intracellular calcium, because the increase in $[Ca^{2+}]_i$ caused by these activators appears quite different in terms of both shape and time course. The increase caused by opening of RyRs appears after 15–30 sec, whereas the increase caused by L-T_3 appears after approximately 100 sec, probably because of the time required for the activation of PLC and for membrane phosphoinositide hydrolysis. Moreover, the increase caused by caffeine/ryanodine lasts much longer. When the hormone is given to cells together with nanomolar ryanodine or caffeine in the millimolar range, the discrete contributions of the two compounds are preserved, indicating that thyroid hormone does not interact with RyRs but only with IP_3 receptors (Fig. 4).

Phosphorylation also contributes to activation of the exchanger in response to thyroid hormone. This is supported by two lines of evidence: 1) a pharmacological approach that depended upon the effects of inhibitors of PKC and MAPK

pathways on thyroid hormone-induced pH_i increase and 2) immunoblotting experiments that showed in thyroid hormone-treated cells both the translocation of PKC-α from cytosol to the plasma membrane and the phosphorylation of MAPK (ERK1/ERK2). The involvement of PKC and MAPK in other nongenomic effects of thyroid hormone in different cell types has previously been reported (13, 16). Similar roles of PKC and MAPK in the activation of the Na^+/H^+ exchanger in freshly isolated chick embryo hepatocytes suggest that common signal-transducing kinase mechanisms might be operative for the nongenomic effects of thyroid hormone on this transport system in different cells (31, 49, 50). On the other hand, studies in one of our laboratories have shown that a nongenomic effect of estrogen on the Na^+/H^+ exchanger in rat aortic smooth muscle cells is mediated by the same PKC and MAPK pathway (51), and a nongenomic effect of aldosterone on the Na^+/H^+ exchanger in MDCK cells mediated by the MAPK pathway has also been reported (52). It is possible that other hormones acting through the phosphoinositide and MAPK pathways may also influence the activity of the Na^+/H^+ exchanger via this kinase-dependent mechanism.

A schematic outline of the sequence of events initiated by thyroid hormones that culminate in modulation of the exchanger is shown in Fig. 8. The cell surface hormone signal is transduced through stimulation of tyrosine kinase activity and consequent activation of PLC; hydrolysis of phosphatidylinositol bisphosphate by PLC yields IP_3 and diacylglycerol (DAG). The DAG activates the classic isoforms of PKC, whereas IP_3 causes an increase in $[Ca^{2+}]_i$ from calcium stores in sarco/endoplasmic reticulum. The rise in $[Ca^{2+}]_i$ also supports PKC activation and its role upstream in the MAPK pathway (16).

Elevation in $[Ca^{2+}]_i$ appears to be important for activation of the Na^+/H^+ exchanger. Regulation of the exchanger depends in several cell types on pathways activated by cellular acidification (38). Our results indicate that the cytoplasmic alkalinization caused by thyroid hormone is due to a calcium-dependent pathway. The finding described here is new, in that this effect is activated not by a growth factor or a peptide hormone, as is usually the case (53), but by thyroid hormone, a factor thought primarily to act through a nuclear hormone receptor. L-6 myoblasts from rat skeletal muscle possess classical nuclear receptors for thyroid hormones (54). The effect of these hormones on the expression of the Na^+/H^+ exchanger units in renal brush border membranes of mammalian kidney cells in culture and on the expression of different isoforms of the exchanger in the renal tubule has been reported (26–29).

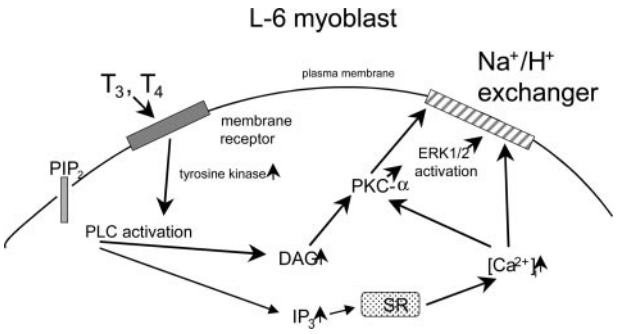

FIG. 8. Scheme of the sequence of events initiated by thyroid hormone leading to the modulation of the Na^+/H^+ exchanger in L-6 myoblasts. The plasma membrane hormone signal is transduced to stimulation of tyrosine kinase activity and consequent activation of PLC; hydrolysis of phosphatidylinositol bisphosphate by PLC yields DAG and IP_3. DAG in turn activates the classic isoforms of PKC, whereas IP_3 causes an increase in $[Ca^{2+}]_i$ from calcium stores in sarco/endoplasmic reticulum. The rise in $[Ca^{2+}]_i$ also supports PKC activation.

We show here a nongenomic cell membrane-initiated effect of iodothyronines. What is the physiological relevance of such an effect of L-T_3 on L-6 myoblasts from rat skeletal muscle? A nongenomic effect of thyroid hormone on the Na^+/H^+ exchanger could contribute to the appropriate functioning of skeletal muscle myofibrils, because an increase in basal activity of the exchange, provided by constant ambient thyroid hormone levels, would support recovery from the acidosis that the muscle develops after the contraction process. Our results with caffeine and ryanodine suggest that the main function of this action of thyroid hormone does not involve the excitation-contraction process, where the RyRs together with the voltage-sensitive receptors are major actors that do not appear to respond to thyroid hormone (results not shown).

In a recent review, we have extended the possible physiological functions of a short-term modulation of the exchanger by thyroid hormone (50) to different cellular activities: exocytosis, enzyme activity, and differentiation. It has been proposed that IP_3 receptor-mediated calcium release plays an important role in different pathways leading either to cell proliferation or cell death (55). In L-6 myoblasts, both events may happen, leading to differentiation from myoblasts to myotubes, a process normally associated with certain events of apoptosis (Incerpi, S., unpublished results). The modulation of pH_i could contribute to discrimination between differentiation or death response, because it is well known, even if not as a general rule, that an increase in pH_i may be a differentiation signal, whereas acidification may be a signal for apoptosis (55). We also propose that the acute nongenomic effects of thyroid hormone that we can see in the experimental models are very likely chronic effects that help to set basal activities of homeostatic pumps and channels in the plasma membrane. This appears particularly relevant, taking into account that alterations in thyroid hormone levels have a dramatic impact not only on the skeletal muscle but also on the myocardium, with consequent alterations of myocardial contractility, speed of relaxation, cardiac output, and heart rate (34, 56).

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