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Biphasic Stimulation of Cellular Calcium Concentration by 3,5,3′-Triiodothyronine in Rat Thymocytes†

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ABSTRACT: 3,5,3′-Triiodothyronine (T_3) produced a rapid and transient increase in ^{45}Ca uptake and cytoplasmic free calcium concentration in rat thymocytes, which is the most rapid effect of T_3 in this system. This effect was manifested in cells suspended in medium containing 1 mM calcium. The T_3 effect on ^{45}Ca uptake was evident at 15-30 s, reached maximum at 30-60 s, and returned to control values at 5 min. The T_3 effect on cytoplasmic free calcium concentration was seen after 30 s, reached maximum at 7 min, and returned to control values after 24 min. In cells suspended in Ca^{2+}-free medium, T_3 produced a similar rapid increase in ^{45}Ca uptake, which was sustained for at least 60 min, but T_3 failed to change cytoplasmic free calcium concentration. Alprenolol (10 μM) blocked the stimulatory effects of T_3 on these two functions in a similar fashion. From these results, I suggest that in rat thymocytes T_3 influences cellular calcium economy through a biphasic mechanism in which T_3 first increases calcium uptake which, in turn, is followed by a release of calcium from intracellular pool(s), resulting in a further increase in cytoplasmic free calcium concentration and the activation of Ca^{2+}-regulated systems. Moreover, the present study provides further support for the postulate that in the rat thymocyte calcium serves as the first messenger for the plasma membrane-mediated stimultory effects of T_3 on several metabolic functions.

In several tissues, such as the heart, it has been postulated that agents exert their calcium-mediated functions through a two-step process. First, the agent acts to increase calcium uptake and thereby cytoplasmic free calcium concentration; such an increase, which is too small to activate the system itself, triggers the release of calcium from intracellular pool(s), primarily the endoplasmic reticulum, which results in a further increase in cytoplasmic free calcium concentration and the activation of the biologic system (Berridge, 1985; Langer, 1976, 1979).

We have shown previously in rat thymocytes that thyroid hormone requires calcium to exert its plasma membrane-

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mediated actions (Segal & Ingbar, 1981, 1984; Segal et al., 1985). To explore further the nature of the role of calcium in this system, the effect of 3,5,3′-triiodothyronine (T_3)[1] on calcium metabolism in the rat thymocyte was examined, employing ^{45}Ca and the quin 2 probe. I demonstrate here that T_3 produced a rapid and transient increase in both ^{45}Ca uptake and cytoplasmic free calcium concentration and propose that the thyroid hormone exerts this action through a biphasic mechanism: an initial increase in extracellular calcium uptake which is followed by an accelerated release of intracellular calcium to increase cytoplasmic free calcium concentration further, which acts, in turn, to activate several calcium-mediated metabolic functions in the cell (Segal & Ingbar, 1979, 1981, 1984; Segal et al., 1985).

Experimental Procedures

Animals used were 25–28-day-old female rats of the Sprague-Dawley strain Crl:CD (SD)BR (Charles River Laboratories, Wilmington, MA).[2]

Thymocytes were isolated according to a procedure described previously (Segal & Ingbar, 1979), and cells were suspended with the standard buffer, pH 7.4, which contains 1 mM calcium (20 mM Tris-HCl, 5 mM Tris base, 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 2.5 mM $MgCl_2$; and 1.5 mM NaH_2PO_4), or in a calcium-free buffer, pH 7.4 (the standard buffer devoid of $CaCl_2$, which contains 5 μM calcium owing to calcium contaminant in the buffer salts; measured by atomic absorption flame photometry with a Perkin-Elmer Model 303 spectrophotometer).

Measurement of ^{45}Ca Uptake. Measurement of ^{45}Ca uptake by rat thymocytes was performed as described previously (Segal & Ingbar, 1984). In short, thymocytes (45×10^6 cells/mL), suspended with the standard or calcium-free medium, were transferred to small plastic tubes (4-mL capacity) placed in a shaking water bath and were equilibrated for 10 min at 37 °C in air. Then, ^{45}Ca (3 μCi/mL, 3.4 μM; specific activity 884 mCi/mmol; New England Nuclear, Boston, MA) was added alone or together with T_3 in various concentrations, and incubation was continued for various periods of time. At the end of incubation, 200-μL aliquots in triplicate were quickly transferred to microtubes and centrifuged at 10000g for 20 s (Beckman microfuge), and radioactivity content in the pellet was measured with a β counter. In parallel experiments, [^{3}H]mannitol (3 μCi/mL, specific activity 17 Ci/mmol; New England Nuclear, Boston, MA) was employed, rather than ^{45}Ca, as an extracellular marker, and the pellet extracellular values were subtracted from total ^{45}Ca values to give the net ^{45}Ca uptake by the cell.

Measurement of Cytoplasmic Free Calcium Concentration. Thymocyte-free calcium concentration was measured by using the quin 2 technique as described previously (Segal, 1986). In short, thymocytes were suspended in the standard or Ca^{2+}-free medium. Two-milliliter aliquots of 1×10^8 cells/mL were transferred into small plastic tubes (4-mL capacity) and were incubated with 50 μM quin 2/AM (the esterified quin 2; Calbiochem-Behring, San Diego, CA) at 37 °C for 20 min. Then, the extracellular indicator was removed by means of centrifugation and washing. The cells were then resuspended with the respective medium and were kept at room temperature. Prior to measurements, an aliquot of 4×10^7 cells was centrifuged at 10000g for 4 s, the supernatant was aspirated, and the cells were resuspended with 2 mL of warm (37 °C) medium and transferred into a cuvette placed in a spectrofluorometer. After a 3-min equilibration period, the different agents in various concentrations and combinations were added to cells, and the change in fluorescence intensity was measured continuously. In comparable experiments with control (T_3-untreated) thymocytes, fluorescence intensity increased gradually with incubation time owing to a slow leakage of quin 2 from the cells of approximately 12% per hour at 37 °C. (Measurements of changes in fluorescence intensity in the cell-free medium revealed that T_3 did not change the leakage rate of quin 2 from thymocytes.) Control values were subtracted from those of the T_3-treated cells to give the net change in fluorescence intensity produced by the hormone. Maximal effect was reached at about 6–8 min after the addition of the thyroid hormone. During the entire measurement period, cells were kept at 37 °C under continuous mixing. Quin 2 fluorescence was measured with the SF-1 Baird spectrofluorometer under the following setting: excitation, 339 nm with a 1.5-nm slit; emission, 492 nm with a 6-nm slit. Cytoplasmic free calcium concentration was measured according to the technique described by Tsien and co-workers (Tsien et al., 1982). Employing this technique, cytoplasmic free calcium concentration in the control thymocytes was found to be 131 nM.

Cell Viability. Cell viability was measured by the trypan blue technique (Segal & Ingbar, 1980a), in which a viable cell is one which excludes the dye. Cell viability, measured before and at the end of each experiment, was the same in the control and the experimental groups, ranging between 90% and 95%.

Statistical Analysis. Where appropriate, statistical analysis of the differences among experimental groups was performed by using the Dunnett's test for comparisons between multiple experimental groups and a single control, and by the analysis of variance followed by the Newman–Keuls multiple range test for comparisons of multiple groups with each other (Zar, 1974).

Results

Effect of Triiodothyronine. Initial experiments were performed with 1 μM T_3, in which the effects of the hormone on ^{45}Ca uptake and cytoplasmic free calcium concentration were examined in thymocytes suspended in standard medium (containing 1 mM calcium) and in Ca^{2+}-free medium (containing 5 μM calcium) (Figure 1).

Standard Medium. T_3 produced a very rapid and transient increase in both ^{45}Ca uptake and cytoplasmic free calcium concentration. The increase in ^{45}Ca uptake was evident already within 15–30 s, reached maximal values at 30–60 s, and then declined rapidly, reaching control values at about 5 min after the addition of the hormone. The T_3-induced increase in cytoplasmic free calcium concentration became evident within about 30 s, reached maximal values at 6–8 min, remained at maximum for an additional 4 min, and then declined gradually, reaching control values only after 24 min.

Calcium-Free Medium. As with the standard medium, T_3 produced the same proportionate rapid increase in ^{45}Ca uptake that reached maximum at 30–60 s, and then declined, reaching rapidly a new equilibration level, above control, and this stimulatory effect of T_3 was sustained for at least 60 min (the longest period examined).[3] In cells suspended with Ca^{2+}-free

[1] Abbreviations: T_3, 3,5,3′-triiodothyronine; 2-DG, 2-deoxyglucose; CFCC, cytoplasmic free calcium concentration; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

[2] Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [*DHEW Publ. (NIH) (U.S.)*, 1978].

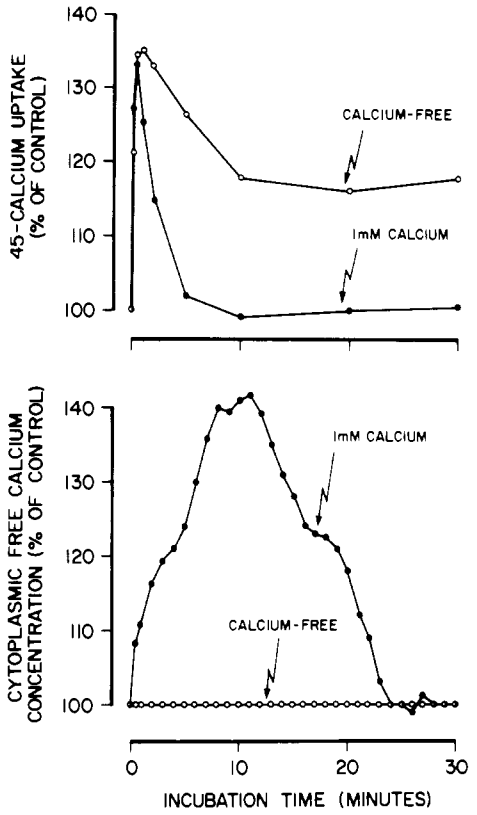

FIGURE 1: Effect of 3,5,3′-triiodothyronine on ^{45}Ca uptake and cytoplasmic free calcium concentration in rat thymocytes. ^{45}Ca uptake (upper panel): Thymocytes were suspended with standard or calcium-free buffer. ^{45}Ca (3 μCi/mL; 3.4 μM) was added alone or together with T_3 (1 μM), and its uptake by the cells was measured after various periods of time. Values shown are the means obtained from one experiment and are typical of those obtained in three other experiments. Cytoplasmic free calcium concentration (lower panel): Quin 2 preloaded thymocytes were suspended with standard or calcium-free buffer. T_3 (1 μM) was added, and its effect on cytoplasmic free calcium concentration was measured continuously for 30 min. Cytoplasmic free calcium concentration in the control thymocytes was found to be 131 nM in cells suspended with the standard buffer and 44 nM in cells suspended in calcium-free buffer. Values shown are the means obtained from a single experiment and are typical of those obtained in two other experiments.

medium (in the presence or absence of 0.1 mM EGTA, reducing the free Ca^{2+} concentration to about 1 nM), T_3 had no effect on cytoplasmic free calcium concentration.

In the ensuing experiments, cells were suspended with the standard medium, and the effects of T_3 on ^{45}Ca uptake and ctyoplasmic free calcium concentration were measured. In studies with ^{45}Ca, cells were incubated with the labeled ion, in the presence or absence of T_3, for 30 s, and in studies of cytoplasmic free calcium concentration, measurements were performed as described under Experimental Procedures.

Concentration Response. The effects of T_3 in various concentrations on thymocyte ^{45}Ca uptake and cytoplasmic free calcium concentration were then examined. T_3 produced a concentration-related increase in both ^{45}Ca uptake and cytoplasmic free calcium concentration (Figure 2). In these effective concentrations, the stimulatory effects of T_3 on ^{45}Ca uptake (Segal & Ingbar, 1984) and on cytoplasmic free calcium concentration follow the same patterns described for 1 μM T_3 in Figure 1 except that values were smaller at the lower T_3 concentrations (maximal increase in cytoplasmic free calcium concentration was seen at about 8 min after the addition of the hormone, values which are shown in Figure 2). Here, as with the other metabolic effects of T_3 in rat thymocytes (Segal & Ingbar, 1979, 1981; Segal et al., 1985), the lowest effective concentration of T_3, when given alone, was 1 nM.[4]

Alprenolol. In rat thymocytes, *l*-alprenolol[5] blocks the Ca^{2+}-dependent effects of T_3 on adenylate cyclase activity, cAMP concentration, and 2-deoxyglucose (2-DG) uptake (Segal & Ingbar, 1981; Segal et al., 1985). Therefore, the effect of *l*-alprenolol on the T_3-induced increase in ^{45}Ca uptake and cytoplasmic free calcium concentration was examined. Alprenolol (10 μM), added together with T_3, blocked in a similar fashion the stimulatory effects of T_3 on both ^{45}Ca uptake and cytoplasmic free calcium concentration (Figure 3). *d*-Alprenolol (0.1 μM–1 mM) was without effect.

Discussion

Our previous studies in rat thymocytes indicate that calcium may serve as the first messenger in the plasma membrane-mediated stimulatory effect of T_3 on 2-DG uptake (Segal & Ingbar, 1979, 1981, 1984; Segal et al., 1985). The present study supports this possibility; showing that in the rat thymocyte the effect of T_3 on cellular calcium uptake and cytoplasmic free calcium concentration is the most rapid effect of T_3 in this system and that alprenolol, which inhibits the effect of T_3 on cellular calcium uptake and cytoplasmic free calcium concentration (Figure 3), inhibits in a similar fashion the stimulatory effects of T_3 on the other Ca^{2+}-mediated metabolic functions: adenylate cyclase activity, cellular cAMP concentration, and 2-DG uptake (Segal & Ingbar, 1980a,b, 1981; Segal et al., 1985).

In cells suspended with the standard medium [containing 1 mM calcium, and in which T_3 increases 2-DG uptake (Segal & Ingbar, 1979)], T_3 produced a very rapid and transient increase in both ^{45}Ca uptake and cytoplasmic free calcium concentration, which was evident within 15–30 s, and then declined and returned to control values. However, the effect

[3] The new steady-state value of cellular ^{45}Ca concentration in the T_3-treated thymocytes, which reflects the equilibrium between ^{45}Ca influx and efflux rates, is lower than the brisk maximal value because of the contribution of ^{45}Ca efflux, which was nil at the beginning of incubation, minute at maximal values, and increased gradually with the increase in cellular ^{45}Ca concentration.

[4] In this system, T_3 produces a Ca^{2+}-dependent increase in 2-DG uptake, an effect that is demonstrable in vivo and has physiological relevance (Segal & Ingbar, 1980a, 1981, 1985). When given alone in vitro, as in the present study, the lowest effective concentration of T_3 to increase 2-DG uptake was 1 nM. It has been postulated that the physiological concentration of T_3 is that of the serum-free T_3 concentration of 3–10 pM calculated from in vitro equilibration measurements. However, recent studies by Pardridge and co-workers (Pardridge & Landaw, 1987; Pardridge & Mietus, 1980) point to the possibility that in vivo the available concentrations of T_3 to the cell surface, and also intracellularly to the nucleus, are much greater than 3–10 pM. These investigators suggest that thyroid hormone, in particular T_3, can reach the tissue also in the serum protein-bound form, as much as 50%, and that the serum protein-bound T_3 is available to the cell by an enhanced dissociation of T_3 at the microcirculatory surface. Hence, there exists an equilibrium between plasma, extracellular, and intracellular pools of T_3, so that the in vivo predictively available (exchangeable) extracellular and cytosolic T_3 concentrations [which are at equilibrium due to a nonactive transport of T_3 through the plasma membrane (Mooradian et al., 1985)] are about 0.5–1 nM. This is consonant with the observations of Oppenheimer and co-workers that the nuclear receptors for T_3 are approximately 50% saturated in vivo and that the binding affinity of the nuclear T_3 receptor is 1 nM (Oppenheimer, 1979). Moreover, in studies on the effect of T_3 on sugar uptake by cultured chick embryo cardiac myocytes, Dickstein and co-workers (Dickstein et al., 1983) have shown that the bound T_3 is as effective as the free T_3 in eliciting a plasma membrane-mediated action. All in all, it is plausible that the physiological concentration of T_3 for plasma membrane-mediated action is much greater than the serum free T_3 concentration measured in vitro by the equilibration technique and that a concentration of 1 nM is within this range.

[5] *d*-Alprenolol was without effect.

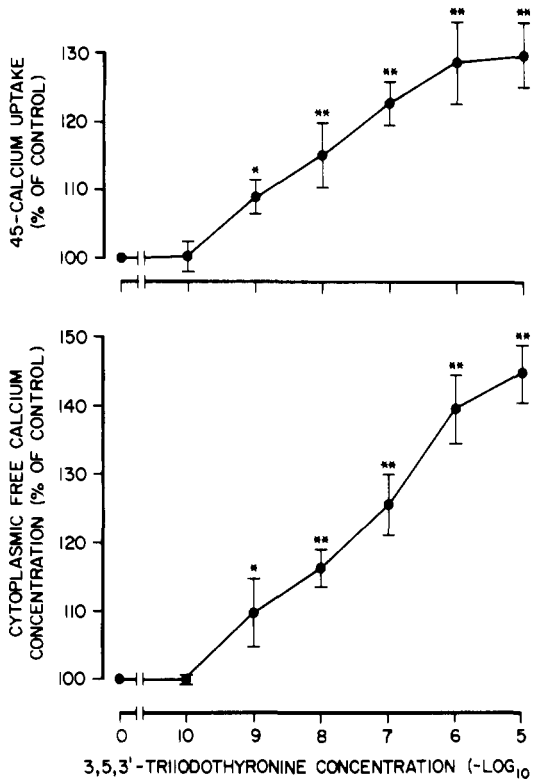

FIGURE 2: Effect of 3,5,3′-triiodothyronine on thymocyte ^{45}Ca uptake and cytoplasmic free calcium concentration: dose–response. Thymocytes were suspended with the standard buffer. The experimental procedure was the same as that described in the legend to Figure 1. ^{45}Ca uptake (upper panel): T_3 was added together with ^{45}Ca, and ^{45}Ca uptake was measured 30 s later. Control values: 1940 ± 152 cpm $(9 \times 10^6$ cells$)^{-1}$ $(30$ s$)^{-1}$. Values shown are the means ± SD obtained from four separate experiments. One and two asterisks indicate significantly greater than control ($p < 0.05$ and $p < 0.01$, respectively). Cytoplasmic free calcium concentration (lower panel): Results shown are those obtained 8 min after the addition of T_3 (maximal effect). Values shown are the means ± SD obtained from seven different experiments. One and two asterisks indicate significantly greater than control ($p < 0.05$ and $p < 0.01$, respectively).

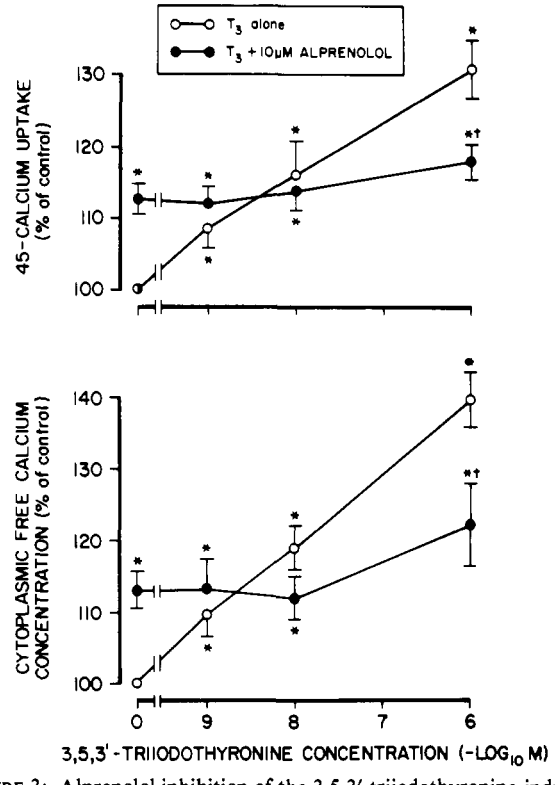

FIGURE 3: Alprenolol inhibition of the 3,5,3′-triiodothyronine-induced increase in ^{45}Ca uptake and cytoplasmic free calcium concentration in rat thymocytes. The experimental procedure was the same as that described in the legend to Figure 2. Where indicated, *l*-alprenolol (10 μM) was added together with T_3. ^{45}Ca uptake (upper panel): Values shown are the means ± SD obtained from 12 separate experiments. Asterisks indicate significantly greater than control ($p < 0.01$). Daggers symbolize significantly smaller than the corresponding T_3-alone group ($p < 0.01$). Cytoplasmic free calcium concentration (lower panel): Values shown are the means ± SD obtained from three separate experiments. Asterisks indicate significantly greater than control ($p < 0.01$). Daggers indicate significantly smaller than the corresponding T_3-alone group ($p < 0.01$).

of T_3 on these two systems followed a different time pattern. The T_3 effect on ^{45}Ca uptake was somewhat more rapid, evident already after 15 s, reached maximum at 30–60 s, and then declined rapidly to reach control values at about 5 min after the addition of the hormone. In contrast, the T_3 effect on cytoplasmic free calcium concentration reached maximum at 7 min and returned to control values only 24 min after the addition of the hormone.

Moreover, in cells suspended with Ca^{2+}-free medium [containing 5 μM calcium, where T_3 does not enhance 2-DG uptake (Segal & Ingbar, 1981)], T_3 produced the same rapid increase in ^{45}Ca uptake as that in cells suspended with the standard medium, but the T_3-induced increase in cellular ^{45}Ca values, after reaching a new equilibration level,[3] was sustained for at least 60 min. Furthermore, in cells suspended with Ca^{2+}-free buffer, T_3 had no effect on cytoplasmic free calcium concentration. This indicates that T_3 affects thymocyte Ca^{2+} concentration through two separate systems: the first, Ca^{2+} uptake, which is activated rapidly and is abolished rapidly and is independent of the physiological concentration of extracellular calcium; and the second, cytoplasmic free calcium concentration, which is elevated promptly, but slower than Ca^{2+} uptake, and remains elevated for a much longer period of time than that of the Ca^{2+} uptake and is dependent on the physiological concentration of extracellular calcium.

The difference between the effects of T_3 on these two systems, ^{45}Ca uptake and cytoplasmic free calcium concentration, in the presence or absence of 1 mM extracellular calcium, could be explained on the basis of a biphasic mechanism of action for the T_3-induced increase in the cytoplasmic free calcium concentration in the rat thymocyte; T_3, that binds to receptors on the plasma membrane (Segal & Ingbar, 1982), initiates its action by increasing extracellular calcium uptake into the cell, an effect that is independent of extracellular calcium concentration (T_3 produced the same proportionate increase in ^{45}Ca uptake in thymocytes suspended with the standard and the Ca^{2+}-free buffer). This results in a small increase in cytoplasmic free calcium concentration which, upon reaching a threshold, triggers the release of calcium from intracellular pool(s) to elevate cytoplasmic free calcium concentration further. The increase in cytoplasmic free calcium concentration acts, in turn and probably through calmodulin, to activate various systems in the cell (Segal & Ingbar, 1979, 1981, 1984; Segal et al., 1985), including the plasma membrane Ca^{2+}-ATPase (Davis et al., 1983; in rat thymocytes, J. Segal et al., unpublished observations); Ca^{2+}-ATPase activity increases with the increase in cellular calcium concentration.

At this point, two opposing systems are in action: one, the Ca^{2+}-ATPase which pumps calcium out of the cytoplasm; and the other, primarily the release of calcium from intracellular pool(s) (most likely endoplasmic reticulum), which acts to increase cytoplasmic free calcium concentration (the contribution of extracellular calcium uptake is small). These two systems counterbalance one another for a short period, during which time cytoplasmic free calcium concentration remains

elevated, and then their activity, along with the cytoplasmic free calcium concentration, starts to decline owing to a gradual depletion of the intracellular calcium pool(s), until the cytoplasmic free calcium concentration reaches control values.

In measurements of ^{45}Ca uptake, the sole source of labeled calcium is extracellular; hence, the increase in Ca^{2+}-ATPase activity results in the expulsion of ^{45}Ca, which is not compensated since ^{45}Ca is not present in the intracellular calcium pool(s), and in a rapid decline in cellular ^{45}Ca values.

In the presence of a low, subphysiological concentration of calcium (5 μM), the proportionate increase in calcium uptake produced by T_3 is about the same as that in the presence of 1 mM extracellular calcium, but the increase in calcium uptake in absolute values is minute so that cytoplasmic free calcium concentration remains below the threshold level and there occurs no release of calcium from the intracellular pool(s) and no activation of Ca^{2+}-ATPase. Thus, no significant change in cytoplasmic free calcium concentration (measured with the quin 2 probe) is apparent, and the elevated values of ^{45}Ca concentration in the T_3-treated cells are sustained.[3]

Further support for this biphasic effect of T_3 on cellular calcium concentration in the rat thymocyte comes from the studies with the β-adrenergic antagonist alprenolol, in which alprenolol blocked in a similar fashion the effects of T_3 on thymocyte ^{45}Ca uptake and on cytoplasmic free calcium concentration (Figure 3).

Registry No. T_3, 6893-02-3; Ca, 7440-70-2; alprenolol, 13655-52-2.

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Lamin B Shares a Number of Distinct Epitopes with Lamins A and C and with Intermediate Filament Proteins†

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ABSTRACT: Four monoclonal antibodies raised against rat liver nuclear lamins and an anti-intermediate filament antibody [Pruss, R. M., Mirsky, R., & Raff, M. C. (1981) *Cell (Cambridge, Mass.) 27*, 419–428] have been used to identify epitopes shared by lamin B with lamins A and C, and with intermediate filament proteins. The antibodies defined two major antigenic regions on the three lamins which were both homologous with mouse epidermal keratins as well as hamster vimentin and desmin. Three distinguishable epitopes shared by lamin B with lamins A and C were identified by competition studies between pairs of antibodies and by reaction against *N*-chlorosuccinimide and cyanogen bromide cleavage fragments. These results support the hypothesis that lamin B, despite important biochemical differences with lamins A and C, shares with them some of the structural characteristics typical of intermediate filament proteins.

The peripheral lamina is an important structural component of nuclei: located between chromatin and inner nuclear membrane, it forms a continuous structure with the pore complexes which can be isolated biochemically while still maintaining the original shape of the nucleus [for recent reviews, see Berezney (1984), Gerace et al. (1984), and Krohne and Benavente (1986)]. The exact molecular details of the interaction between elements of the lamina and pore complexes as well as between lamina and chromatin or nuclear membrane are not well understood. Peripheral lamina appears to be exclusively composed of polypeptides called lamins, varying in number from 1 to 3 according to species and cell type (Krohne & Benavente, 1986).

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