# Increased Sodium Influx and Calcium Uptake in Erythrocytes in Hyperthyroidism: Role of Abnormal Membrane Lipid Levels

Carlo De Riva, Flavio Virgili, and Francesco Frigato

**The study was designed to examine the effects of thyroid hormones on red blood cell (RBC) membrane phospholipids and ion transport. We demonstrated that in untreated Graves' disease, an alteration in the phospholipid pattern is present at cellular levels, with a concomitant derangement in membrane permeability defined as $^{22}Na$ influx and $^{45}Ca$ uptake. Thionamide therapy replaced the normal membrane permeability, presumably as a consequence of restoring the normal phospholipid membrane composition. We conclude that thyroid hormones are able to induce a quick breakdown of a large number of membrane components such as membrane phospholipids.**


IT HAS BEEN REPORTED that thyroid hormones are able to reduce $Na^+K^+$-adenosine triphosphatase (ATPase) activity in red blood cells (RBCs)[1,2] and that inhibition leads to an increase in intracellular sodium concentration.[2,3] However, the mechanism by which thyroid hormones inhibit the Na-K pump is not clear.[3] It has been suggested that thyroid hormones inhibit the synthesis of the sodium pump during maturation in the bone marrow,[3,4] whereas other studies suggest that these effects depend on a rapid breakdown of a large number of membrane compounds such as enzymes, membrane phospholipids, or receptors in the mature circulating cells.[5,6] It has also been demonstrated that the activity of $Na^+K^+$-ATPase, normally effective in RBCs, is provided by a regulatory system that requires a rapid basal phosphatidylinositol (PI) membrane turnover, and it has been supposed that an impaired $Na^+K^+$-ATPase activity could be a consequence of the reduction of membrane PI.[7,8]

In patients affected by untreated hyperthyroidism, there is evidence of a defect not only in the activity of $Na^+K^+$-ATPase in RBCs, but also in sodium transport and, as indicated earlier, in intracellular sodium concentration.[2,3] Since thyroid hormones accelerate the breakdown of a large number of membrane proteins,[7,9,10] it has been suggested that the effect of an increase in circulating thyroid hormones on the erythrocyte sodium pump is part of a generalized effect on membrane structure.[11] It should be noted that any modification in the lipid bilayer may alter the function of membranes, and some disorders of cellular physiology have been associated with abnormal membrane lipid composition, such as cation transport defects[12] or any alteration in the receptor-mediated signaling pathway.[13] In light of these considerations, it is interesting that thyroid hormones may act on the signal-transducing phosphoinositide and that the binding of thyroid hormones to plasma membranes may be regulated by the amount of specific inositol phosphates available.[14]

Little information is currently available not only about this issue, but also about the physiology of RBC ion transport in hyperthyroidism, especially with regard to the effects of thyroid hormones on membrane lipid structure and consequently on cell permeability, since any defect in membrane organization could modify cation transport systems.[13-15]

With regard to these observations, we studied $Na^+$ influx and $Ca^{2+}$ uptake in erythrocytes from patients with hyperthyroidism and investigated the possible existence of a relationship between ion transport defects and phospholipid membrane composition before and after a specific period of antithyroid treatment, to elucidate whether the effect of thyroid hormones on cation transport is part of a generalized effect on the erythrocyte membrane.

## SUBJECTS AND METHODS

### *Patients and Controls*

We studied 20 hyperthyroid patients being treated at the Endocrinology Department of our hospital (17 women and three men; mean age, 26 ± 4.2 years). All were clinically hyperthyroid from Graves' disease, as confirmed by plasma thyroid hormone tests, antibody determinations, thyroid scans, and clinical observations. Evaluation of RBC parameters ruled out microcytic or macrocytic anemia. A control group contained 20 normal volunteers (17 women and three men; mean age, 27 ± 5.1 years) who were clinically and biochemically euthyroid and were not on any medication. Informed consent was obtained from all subjects.

The experiment was performed in a double-phase comparative study. The first phase (study 1) was performed in a condition of hyperthyroidism, followed by a second determination after a period of 30 days (study 1 + 30 days) from the beginning of antithyroid therapy, with the aim being to clarify whether the observed effects were originated by changes in existing erythrocytes or whether it was necessary to obtain a completely new population of RBCs. The second phase (study 2) took place after 300 days in a condition of persistent euthyroidism (at least from 120 days) obtained with antithyroid treatment using thionamide therapy (Metimazole-Tapazole 5 to 15 mg/d; Eli Lilly, Florence, Italy). This period was considered necessary to obtain a new population of RBCs reaching maturity in a condition of euthyroidism.

From 2 weeks before each study onward, everyone had a constant sodium and potassium intake (120 and 60 mmol/L/d, respectively).

### *Materials*

Plasma total thyroxine ($T_4$) level was measured by an enzyme immunoassay (Syva Emit; Bracco, Milan, Italy). Plasma free $T_4$

*From the Department of Endocrinology, Umberto I° General Hospital, Mestre-Venice, Italy.*
*Submitted February 6, 1995; accepted December 4, 1995.*
*Address reprint requests to Carlo De Riva, MD, Department of Endocrinology, Umberto I° General Hospital, Via Circonvallazione, 10 30170 Mestre-Venezia, Italy.*

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($FT_4$) and free triiodothyronine ($FT_3$) were determinated by radioimmunoassay using a commercial kit from Diagnostic Products (Techno Genetics, Milan, Italy). Thyroid-stimulating hormone (TSH) was determinated by immunoradiometric assay using commercial kits (Ares-Serono, Aubonne, Switzerland).

### *Methods*

All subjects were studied after an overnight fast. Venous blood (20 mL) was collected from an antecubital vein for erythrocyte studies. Plasma was separated from heparinized blood by centrifugation at 1,000 × *g* for 20 minutes and further centrifugation at 3,000 × *g* for 10 minutes to eliminate remaining white blood cells and platelets.

### *Erythrocyte Studies*

*Membrane phospholipids.* RBCs were separated from plasma by centrifugation and washed three times in isotonic saline solution (pH 7.4). RBC membrane lipids were extracted with isopropyl alcohol and chloroform.[16] Phospholipids were separated by two-dimensional thin-layer chromatography on Silica-Gel H (Merck, Rahway, NJ) developed with the solvent system, chloroform-methanol-acetic acid-water (25:15:4:2 vol/vol)[17]; the various phospholipid spots were compared with authentic standards and then quantified by assaying inorganic phosphate using the method of Vaskovsky.[18]

*Sodium influx.* This was examined in the washed RBCs. They were suspended in a 0.145-mol/L Na/K phosphate-buffered saline solution (pH 7.4) with glucose (250 mg/dL), and the hematocrit was adjusted to 30%. The RBCs were incubated with $^{22}Na$ (NEZ-081; New England Nuclear, Boston, MA) at 37°C for 2 hours and then washed three times at 4°C with a 0.1-mol/L $MgCl_2$ solution by centrifugation at 1,200 × *g* for 2 minutes. Radioactivity of $^{22}Na$ remaining in the erythrocytes was determined by a gamma counter.[12,19]

*Calcium uptake.* RBCs were washed four times with an isotonic saline solution (pH 7.4) containing 5 mmol/L KCl and 20 mmol/L imidazole HCl, and the hematocrit was adjusted to 30%. The washed RBCs were preincubated at 37°C for 90 minutes with an isotonic imidazole HCl–buffered saline solution (pH 7.4) of 2 mmol/L iodoacetate and 10 mmol/L inosine, to deplete intracellular adenosine 5′-triphosphate (ATP).[20] One milliliter of ATP-depleted RBCs were incubated with 1.5 mmol/L $^{45}CaCl_2$ (1 μCi/mL) at 37°C for 2 hours. The incubation was terminated by washing the RBCs with ice-cold saline solution four times, and radioactivity ($^{45}Ca$) in RBCs was determined by liquid scintillation counting.

*Intracellular Na-K concentration.* RBCs were washed four times with ice-cold $MgCl_2$ solution (110 mmol/L), and the cells were hemolyzed. Na and K concentrations in the hemolysates were determined by flame emission spectrometry.[19] The precision of the assay (expressed as the coefficient of variation) determined by analysis in duplicate was 3.1% for $^{22}Na$ influx,[19] 2.0% for $^{45}Ca$ uptake,[20] and 2.5% for phospholipid determinations.[17,18] Statistical analysis was performed using a *t* test for paired data ($P < .05$ considered statistically significant).

Correlations were obtained by simple regression analysis.

## RESULTS

### *Study 1*

In hyperthyroid subjects, plasma $T_4$, $FT_4$, and $FT_3$ were increased compared with values in the control group (plasma $T_4$, 218 ± 23.7 *v* 82 ± 13.2 nmol/mL, $P < .01$; $FT_4$, 53.9 ± 9.1 *v* 8.1 ± 1.6 pg/mL, $P < .01$; $FT_3$, 24.8 ± 1.4 *v* 3.8 ± 0.6 pg/mL, $P < .01$, respectively). In the same group, TSH was reduced ($< 0.1$ *v* 2.4 ± 0.9 μU/mL, $P < .01$).

Intracellular sodium concentration was significantly increased in all patients (8.87 ± 1.4 *v* 5.7 ± 0.9 mmol · $L^{-1}$ RBC, $P < .01$), but we did not observe any significant difference in intracellular potassium content (100 ± 9 *v* 99 ± 14 mmol · $L^{-1}$ RBC, $P$ = NS).

$^{22}Na$ influx and $^{45}Ca$ uptake were increased in our patients as compared with the normal volunteers ($^{22}Na$ influx, 2.2 ± 0.21 *v* 1.33 ± 0.16 mmol/L RBC/h, $P < .01$; $^{45}Ca$ uptake, 45 ± 13 *v* 23 ± 7.2 mmol/mL RBC/2 h, $P < .01$).

Among the major components of membrane lipids, phosphatidylcholine and PI were diminished but lysophosphatidylcholine (LPC) was increased in hyperthyroid patients. We did not identify any change in phosphatidylethanolamine, phosphatidylserine, or sphingomyelin (Fig 1).

After 30 days from the beginning of therapy, there were no significant changes in membrane phospholipid composition or ion-transport parameters. Thyroid hormone levels decreased, but were statistically higher than levels in normal volunteers. Nevertheless, the values for circulating TSH were depressed (Table 1).

### *Study 2*

Thionamide therapy restored a normal level of $FT_4$ and $FT_3$ in all subjects. TSH also improved, but did not reach a normal level (0.4 ± 0.2 *v* 2.5 ± 0.8 μU/mL, $P < .01$). We observed a simultaneous decrease in intracellular sodium content and $^{22}Na$ influx and $^{45}Ca$ uptake in erythrocytes (Table 1). There was normalization of the membrane phospholipid composition (Fig 1). When all the subjects were examined, $^{22}Na$ influx and $^{45}Ca$ uptake were correlated inversely with phosphatidylcholine and directly with LPC (Figs 2 and 3). There were no correlations between ion transport parameters and PI.

All results and statistics are summarized in Table 1.

## DISCUSSION

The physiology of RBCs in hyperthyroid patients is distinguished by an occasionally reduced life span that is not present in all cases.[21] The most common RBC abnormality encountered in hyperthyroidism is microcytosis, which was not evident in our patients.[22] Occasionally, thyroid hormone excess produces an increase in RBC mass rather than a decrease, but polycythemia is not usually found.[22]

Little information is currently available on ion transport across the RBC membrane during hyperthyroidism. We have stated that in hyperthyroidism, a biochemical lesion is present in the membrane of RBCs consisting of a marked alteration of membrane lipid composition, coinciding with an increase in $^{22}Na$ influx and $^{45}Ca$ uptake. Finally, in a state of hyperthyroidism, intracellular sodium concentration is increased.

On the other hand, we observed that an adequate period of antithyroid therapy with thionamide restored the normal phospholipid membrane composition in erythrocytes and the normal intracellular sodium concentration.

The reason whereby thyroid hormones are capable of

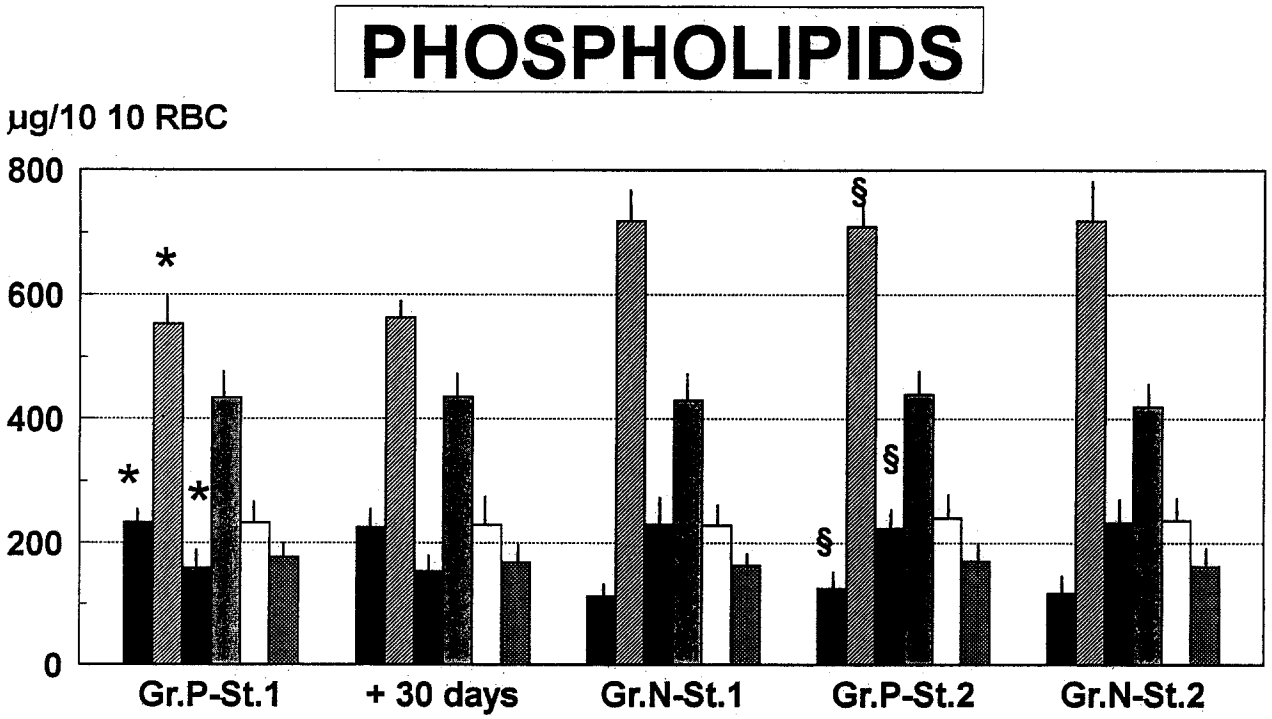


**Fig 1. LPC, phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM) levels in RBC membrane in hyperthyroidism (Gr.P-St.1), after a period of 30 days (+30 days), and after thionamide therapy in euthyroidism (Gr.P-St.2). Gr.N-St.1 and Gr.N-St.2, normal subjects during study 1 and study 2, respectively. *$P < .01$, Gr.P-St.1 *v* Gr.N-St.1 and *v* Gr.N-St.2; §$P < .01$, Gr.P-St.2 *v* Gr.P-St.1 and *v* +30 days.**

modifying the lipid organization of the membrane is obscure. We suggest that it may be considered a direct consequence of increased $FT_3$, which causes a breakdown of membrane phospholipids and consequently an alteration in sodium influx and calcium uptake. The degradation may occur in the circulation during the aging of RBCs or during the maturation in bone marrow. Alternatively, we suggest that the abnormal phospholipidic pattern could be an outcome of an increased activity of phospholipase $A_2$ ($PLA_2$) determined by the increased levels of thyroid hormones. This enzyme degrades glycerophospholipids at the *sn*-2-acyl-glycerol ester linkage, generating stoichiometric amounts of free fatty acid and lysophospholipids. The lysophospholipids generated by $PLA_2$ action remain in the membrane while free fatty acids shift to the circulation. The reduction of PI in the membranes is of particular interest, since it has been established that inositol phosphate is involved in numerous biochemical processes: it regulates the abundance of sites available for the binding of thyroid hormone to human RBC membranes,[14] and the PI pathway is implicated in the transduction of a large number of endocrine signals.[23-26]

A reduction of membrane PI can determine a decline in the intracellular formation of inositol trisphosphate, whereas ($InsP_3$) is formed by the hydrolysis of phosphatidylinositol 4,5-bisphosphate, generated in the membrane by two-stage phosphorylation of PI as a result of the activity of two different specific kinases. It is likely that the formation of InsP3 is the major signal for activation of ion channels.[27] Although we did not observe any correlation between PI and $^{22}Na$ influx or $^{45}Ca$ uptake we cannot rule out the hypothesis that the reduction of PI modifies the permeabil-

**Table 1. Summary of Results**

| | Study 1 | | Study 1 + 30 days | | Study 2 | |
|---|---|---|---|---|---|---|
| | Normals | Patients | Normals | Patients | Normals | Patients |
| Plasma $T_4$ (nmol/mL) | 82 ± 13.2 | 218 ± 23.7* | 79 ± 14 | 152 ± 28* | 80 ± 14 | 96 ± 21.3 |
| $FT_4$ (pg/mL) | 8.1 ± 1.6 | 53.9 ± 9.1* | 8 ± 1.9 | 25.6 ± 10* | 8.2 ± 1.4 | 10.1 ± 1.3† |
| $FT_3$ (pg/mL) | 3.8 ± 0.6 | 24.8 ± 1.4* | 3.6 ± 0.8 | 9.2 ± 0.5* | 3.7 ± 0.7 | 4.2 ± 0.9† |
| TSH (µU/mL) | 2.4 ± 0.9 | <0.1* | 2.5 ± 1 | 0.1* | 2.5 ± 0.8 | 0.4 ± 0.2*† |
| Na RBC (mmol · $L^{-1}$ RBC) | 5.7 ± 0.9 | 8.87 ± 1.4* | 5.6 ± 1.1 | 8.79 ± 1.7* | 5.77 ± 0.8 | 5.66 ± 0.7† |
| K RBC (mmol · $L^{-1}$ RBC) | 99 ± 14 | 100 ± 9 | 101 ± 18 | 97 ± 13 | 97 ± 16 | 97 ± 14 |
| $^{22}Na$ influx (mmol/L RBC/h) | 1.33 ± 0.16 | 2.2 ± 0.21* | 1.34 ± 0.13 | 2.19 ± 0.17* | 1.33 ± 0.14 | 1.34 ± 0.18† |
| $^{45}Ca$ uptake (nmol/mL RBC/2 h) | 23 ± 7.2 | 45 ± 13* | 25 ± 8 | 47 ± 10* | 23 ± 8.4 | 25 ± 11† |

*$P < .05$, hyperthyroid patients *v* normals.

†$P < .05$, hyperthyroid patients in study 1 *v* hyperthyroid patients in study 2.

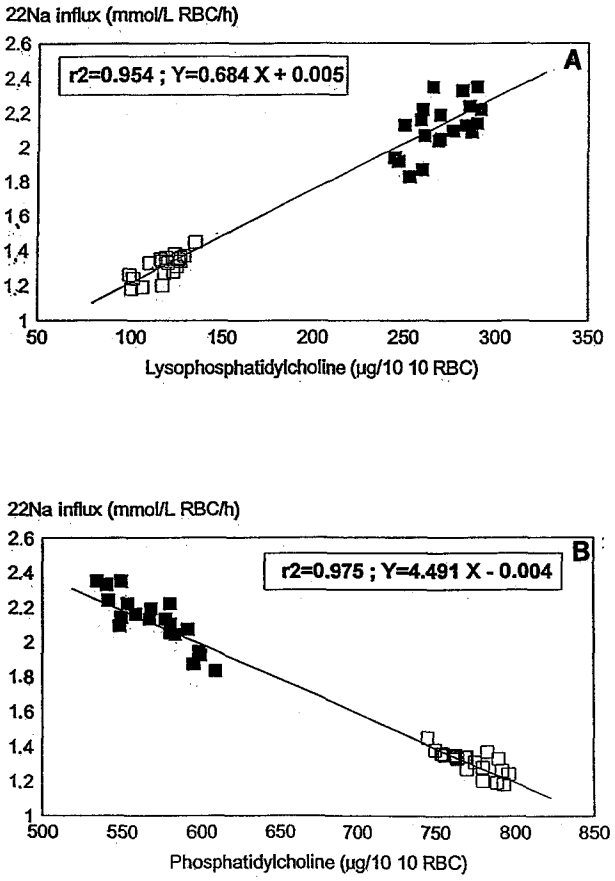


**Fig 2. Linear regression between $^{22}Na$ influx in RBCs and LPC (A) and phosphatidylcholine (B). $P < .05$. (■) Before therapy; (□) after therapy.**

ity of membranes per se. Furthermore, the possibility that the reduction of the PI pathway may cause a modification in cell-surface receptor number and affinity, thereby producing an alteration in cell responsivity to endogenous signals,[13,24,25] cannot be excluded. Since we did not test $Na^+K^+$-ATPase activity, we cannot speculate about the relationship between such enzymatic activity and the amount of membrane PI.[7,8] Furthermore, it is worth noting that hyperthyroid patients show an abnormality in sodium transport, displayed as an increase in $^{22}Na$ influx. Normally, the increased passive entry of sodium may be compensated for by an increase in the maximal rate activity of the $Na^+K^+$ pump (ATP-dependent).[19] Since the activity of $Na^+K^+$-ATPase in RBCs is impaired in hyperthyroid patients,[1-3] this would in turn lead to an elevation of intracellular $Na^+$ concentration. Moreover, considering that the Na-K pump is the major mechanism controlling net accumulation of $K^+$ in numerous tissues, it is reasonable to suppose that these modifications could produce large changes in the small pool of $K^+$ or in osmotically active solutes that could interfere with excitability in many tissues. Although we did not verify any changes in intracellular $K^+$, it is undeniable that, overall, this situation compromises the electrochemical homeostasis on both sides of the plasma membrane.

On the other hand, it is interesting that a persistent increase in intracellular sodium concentration itself stimulates an increase in activity of the membrane sodium pump ($Na^+K^+$-ATPase) and depletes ATP, augmenting energy expenditure and oxygen consumption.[7,8,28]

The absence of a normalization of membrane phospholipid composition after 30 days of methimazole therapy and, on the contrary, the observation of a normal membrane phospholipid pattern tested in a new population of RBCs after correction of the hyperthyroid state seems to rule out the possibility of a direct effect of $T_3$ on membrane composition, and it appears to confirm that the action of $T_3$ requires the presence of nuclear receptors. However, we cannot exclude the hypothesis that the progressive reduction of $FT_3$ could be followed by replacement of a normal membrane lipidic pattern, thereby indicating a direct effect of $T_3$ on membranes.

To obtain further details about the role of membrane phospholipids in the regulation of cation transport, we are currently studying the rate of phospholipid synthesis and turnover in RBCs (and in nucleated cells, ie, macrophages) in hyperthyroidism in reaction to a sodium and calcium load. Although erythrocytes are unnucleated cells and the acknowledged effects of the thyroid hormones occur at the

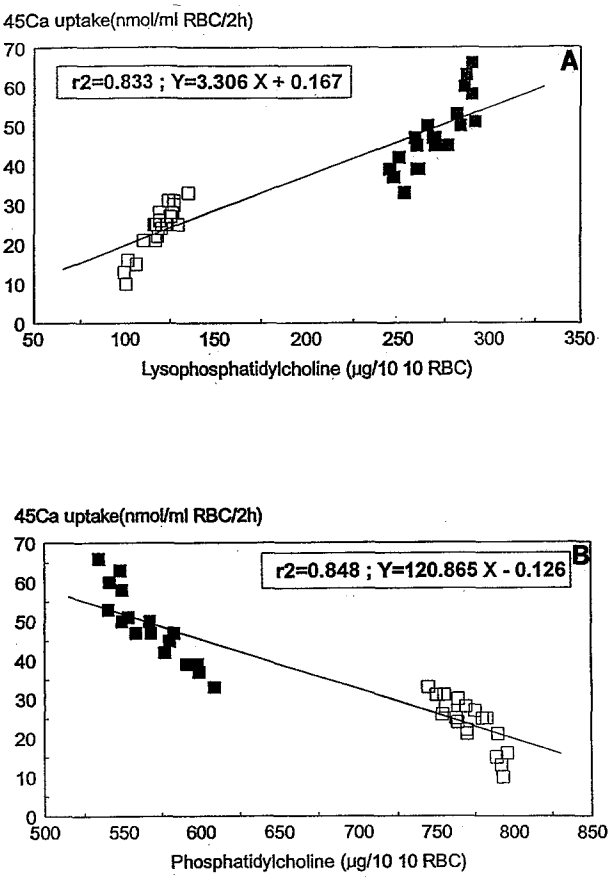


**Fig 3. Linear regression between $^{45}Ca$ uptake in RBCs and LPC (A) and phosphatidylcholine (B). $P < .05$. (■) Before therapy; (□) after therapy.**

nuclear level (in the case of erythrocytes, during maturation in the bone marrow, after which RBCs lose their nucleus), these data are useful for clarifying any property of hyperthyroidism, such as the peripheral effects of thyroid hormone excess.

In summary, membrane transport (Na influx and Ca uptake) in RBCs in hyperthyroid patients is impaired, as is membrane phospholipid composition. The possible association between lipid composition and ion flux phenomena requires further study to clarify whether a link can be established between them. These results may contribute to a better understanding of the pathophysiology of hyperthyroidism and, above all, the effects of thyroid hormones at membrane levels.

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