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Dimethylsphingosine increases cytosolic calcium and intracellular pH in human T lymphocytes

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Abstract

N,N-Dimethyl-D-erythro-sphingosine (DMS) is the *N*-methyl derivative of sphingosine; both are activators of sphingosine-dependent protein kinases. The aim of this work was to study the effect of DMS on cytosolic calcium and intracellular pH (pHi) in human T lymphocytes. The variations of calcium and pH were determined by fluorescence digital imaging using Fura-2-AM and BCECF-AM, respectively. DMS increased both pHi and Ca^{2+}-cytoslic in human T lymphocytes. These effects were dose-dependent. This drug induced a fast increase in pHi and a release of calcium from different intracellular calcium pools than thapsigargin. DMS also induced a Ca^{2+}-influx different from the store-operated calcium channels, since drug effect was not modified by 30 μM SKF 96365. The influx of calcium induced by DMS was completely blocked by preincubation in the presence of nickel, or lanthanum, while the increase in pHi was no affected. However, the presence of cadmium reduced but does not block Ca^{2+}-influx. The inhibition of G-protein by 100 ng/mL pertussis toxin, and the inhibition of tyrosine kinases by genistein significantly reduced the cytosolic calcium increase induced by DMS by an inhibition of both, release of calcium from intracellular pools and influx from extracellular medium. The inhibition of pools emptiness by these drugs was related with the inhibition that they induce in the DMS cytosolic alcalinization. In summary, DMS increases pHi and as consequence releases calcium from intracellular pools, and it increases calcium-influx through a channel different from store-operated channel (SOC). Both cytosolic calcium and pHi increase are modulated by G-proteins and tyrosine kinases.

Keywords: T lymphocytes; *N,N*-Dimethyl-D-erythro-sphingosine; Calcium; pHi tyrosine kinase; G-proteins; Fura-2; BCECF

1. Introduction

Sphingolipid metabolites have an important role as cellular modulators. They are involved in signal transduction mechanisms as calcium-influx [1], or kinase phosphorylation [2,3]. But they are also implicated in cell differentiation and apoptosis [4]. Sphingosine is a product derived from sphingolipid metabolism with multiple intracellular functions. Its low level increases after cellular activation as a consequence of the action of an agonist such as platelet derived growth factor or insulin-like growth factor [5,6]. The effect is an activation of ceramidase that converts ceramide to sphingosine. Sphingosine is then transformed into its methylated derivative DMS [2]. DMS and sphingosine are found naturally in cells, and the enzymes that convert sphingosine to DMS have been detected in several tissues [7–9], which suggests that both are physiological mediators and can be involved in cellular signaling. In this sense DMS induces an important phosphorylation in cells by activating a sphingosine-dependent protein kinases [10], or the contrary effect by inhibiting a sphingosine kinase [11].

Several papers report the effect of sphingolipid derivatives in cytosolic calcium levels. Sphingosine 1-phosphate, produced from sphingosine by the sphingosine kinase, is involved in calcium mobilization from intracellular pools [12]. In the cell line HL-60 sphingosine 1-phosphate has been described as a second messenger, because after the cell activation, sphingosine 1-phosphate increases the mobilization of intracellular calcium [13]. In the same cell line sphingosine and DMS have been described as

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Abbreviations: DMS, *N,N*-dimethyl-D-erythro-sphingosine; pHi, intracellular pH; Tg, thapsigargin; SOC, store-operated channel.

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activators of calcium-influx from extracellular medium [14]. On the other hand, some sphingosine derivatives induced an inhibition on the store-operated calcium-influx in RBL-H3 cells [1] and HL-60 cells [14], while in Jurkat human leukemia T cells, sphingosine releases calcium from intracellular pools [15]. Therefore, depending on the cellular model studied, sphingosine derivatives induce different and even contrary effects. Although the effects of sphingosine 1-phosphate and sphingosine in cytosolic calcium have been extensively described, the mechanisms by which calcium is mobilized are controversial, and only few papers describe some effect of DMS on cytosolic calcium.

Regulation of pHi is crucial to different cellular functions. Changes in pHi take place in response to growth, tumoral promoters, secretory processes or changes in membrane conductance or permeability. Na^+/H^+ exchange and different CO_3H^- transporters are the main mechanisms that cells use to control pHi levels [16]. The modulation of cellular pH is associated in a complex way to cellular function. Several studies show that the cellular response to a variety of growth and vasoactive factors was associated with a cytosolic alkalinization [17]. Some sphingosine derivatives, sphingosylphosphorylcholine and sphingosine 1-phosphate, induce an intracellular alcalinization often associated to Na^+/H^+ exchange, while sphingosine has been described as an inhibitor of Na^+/H^+ exchange activated by phorbol esters [18–21]. However, there are no data about DMS effect on cytosolic pH and the relationships of this parameter with cytosolic calcium.

Ceramides may play a role in modulating immune and inflammatory responses of lymphocytes since these compounds mediate in TNF-α action and enhance IL-2 secretion in these cells [22]. Therefore, it is very important to know the intracellular signals involved in ceramide-mediated response in T healthy cells. It is known that intracellular calcium is an important early signal in lymphocytes, and in previous papers, we did perform several studies to determine the relationship between cytosolic calcium and the cellular modulators pHi and membrane potential in human lymphocytes [23,24]. In addition, in the context of some prolactin studies, we described an important cytosolic calcium increase induced by DMS in human T lymphocytes [25]. Therefore, the aim of this work is to study and characterize the effect of DMS on cytosolic calcium and pHi in human T lymphocytes.

2. Materials and methods

2.1. Chemicals

N,*N*-Dimethyl-D-erythro-sphingosine, thapsigargin (Tg), genistein, and SKF 96365, were from Alexis Corporation. Fura-2 AM was from Molecular Probes. Monoclonal antibody to human CD3, clone UCHT1 was from Serotec. Ethylenediaminetetracetic acid (EDTA), ethilen-glicol-bis(b-aminoethylether) *N*,*N*,*N*′,*N*′-tetracetic acid (EGTA), nickel, cadmium, lanthanum and nigericin were from Sigma. Percoll® was from Pharmacia. Pan T cell Isolation kit was from Miltenyi Biotec.

Concentrated stock solutions (over 1000 times) were always made in vehicle dimethylsulfoxide (DMSO). Control experiments of vehicle were done and no effect was observed, as it is showed in Fig. 1A and B.

2.2. PBS solution

The composition of PBS plus EDTA solution was (mM): Na^+, 153.4; Cl^-, 140.2; HPO_4^{2-}, 8.2; $H_2PO_4^-$, 1.5; K^+, 4.7; EDTA, 2 mM adjusted to pH 7.4.

The composition of physiological saline solution was (mM): Na^+, 142.3; K^+, 5.94; Ca^{2+}, 1; Mg^{2+}, 1.2; Cl^-, 126.2; HCO_3^-, 22.85; HPO_4^{2-}, 1.2; SO_4^{2-}, 1.2. In all the experiments, the incubation medium was equilibrated with CO_2 and the final pH was adjusted to 7.4 prior to use. When the medium was a calcium-free medium, we remove calcium, and we check the calcium concentration of water, and it was lower than 2 μM approximately.

2.3. Human lymphocytes isolation

Peripheral human lymphocytes were isolated from heparinized human blood diluted 1:1 with PBS plus EDTA 2 mM by centrifugation through 57.5% isotonic Percoll®. Percoll® was eliminated by washing three times with PBS plus EDTA 2 mM at 400 *g* for 5 min. Lymphocytes purity was always higher than 80%.

T cell were purified from this population with a Pan T cell Isolation kit, that is an indirect magnetic labeling system for the isolation of untouched T cells; T cell purity was always higher than 90%. Assessment of cell purity was performed by flow cytometry by using an anti-CD3 labeled with FITC.

2.4. Measurement of cytosolic-free calcium and pHi

2.4.1. Image processing

Purified T lymphocytes were loaded with Fura-2 AM (2 μM) and BCECF (0.1 μM) for 10 min at 37°. Loaded cells were washed three times (400 *g*/2 min) and allowed to attach to poly-L-lysine-coated 22-mm glass coverslips for 10 min. The glass coverslips were inserted into a thermostated chamber (Life Science Resources) and cells were viewed with a Nikon Diaphot 200 microscope equipped with epifluorescence optics (Nikon 40X-inmersion UV Fluor objective). The chamber thermostated at 37° was used in the open bath configuration and additions made by aspiration and addition of fresh bathing solution. Intracellular calcium concentration and pH were obtained from the images collected by quadruple excitation fluorescence with a Life Science Resouces equipment. The light source was a

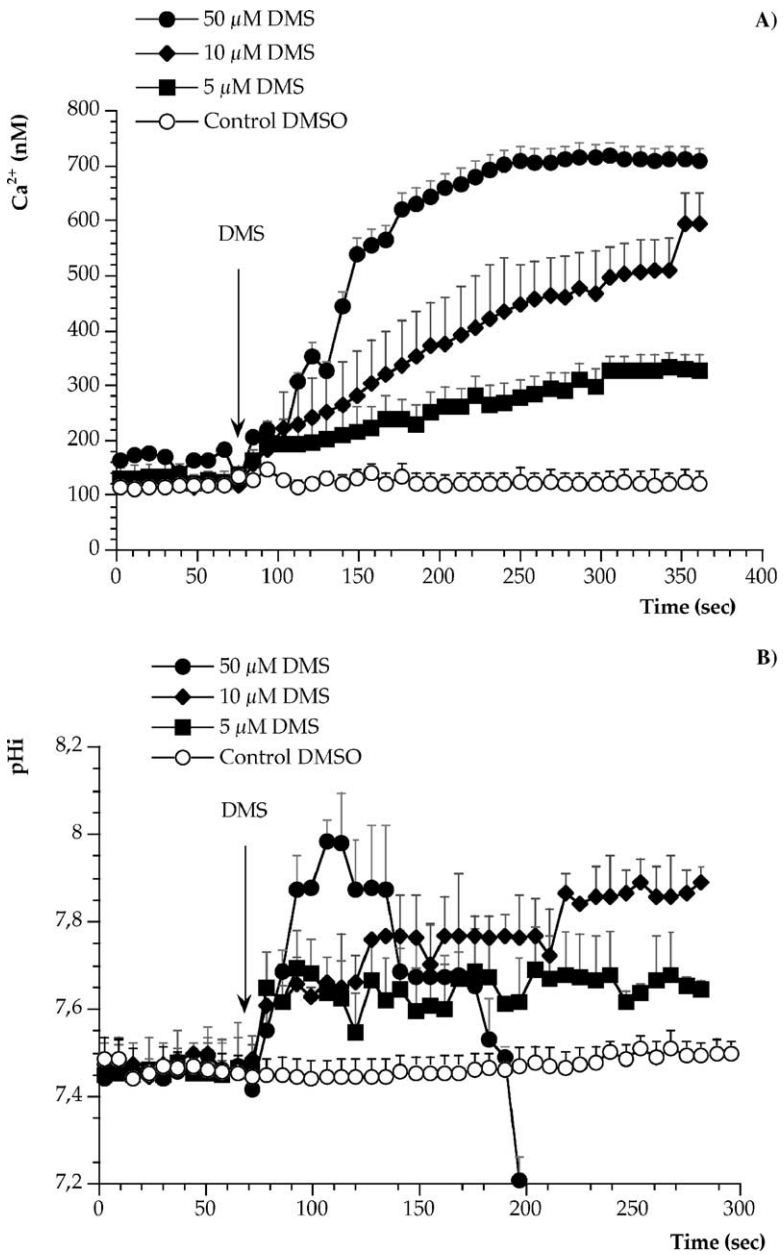

Fig. 1. Effect of different DMS concentrations on cytosolic Ca^{2+} (A) and pHi (B) levels in human T lymphocytes in a calcium-containing medium. The arrow indicates DMS addition of 50 μM (circles), 10 μM (rhomb), 5 μM (squares), or dimethylsulfoxide (open circles). The data are the average of 700, 755, 734 and 640 cells, respectively. Mean ± SEM of five experiments.

175-W xenon lamp, and light reach the objective with optic fiber. The excitation wavelengths for Fura were 340 and 380 nm, with emission at 505 nm, and for BCECF 440 and 490 nm, both for excitation and 530 for emission. The calibration of the fluorescence vs. intracellular calcium was made by using the method of Grynkiewicz *et al*. [26]. The calibration of fluorescence vs. pHi was made using nigericin in K^+ solution as per Thomas *et al*. [27]. Briefly, a calibration curve was obtained with four known values of pH, measuring the fluorescence ratio obtained in the presence of nigericin for each pH value. With these values we obtained in each experiment a calibration curve (ratio vs. pH) which was used to transform any ratio value to pH.

2.5. Statistical analysis

Results were analyzed using the Student's *t*-test for unpaired data. A probability level of 0.05 or smaller was used for statistical significance. Results were expressed as the mean ± SEM.

3. Results

As we described in a previous paper [25], DMS induces an increase in cytosolic calcium levels in human T cells. In the present paper, we characterized this increase and we

checked the effect of DMS on pHi, since variations on cytosolic calcium and pH are related in cellular activation. We first checked the effect of different DMS concentrations in cytosolic calcium and pHi. Fig. 1A shows intracellular calcium levels in the presence of 5, 10 and 50 μM DMS. As shown, DMS induces a dose-dependent increase in cytosolic calcium. Cytosolic calcium increases from 100 to 300, 500 or 700 nM depending on DMS concentration added. As Fig. 1B shows either 10 or 5 μM DMS increase pHi. Fifty micromolar DMS induces a higher pH increase, however, after 2 min cytosolic pH fall to values below 7.2. That is, DMS induces a dose-dependent increase in cytosolic calcium and pH. As 5 μM DMS induces an enough and important effect we choose this concentration to characterize the calcium-influx and the pHi increase.

We checked the effect of DMS in intracellular calcium pools and the importance of these pools in Ca^{2+}-influx and pHi. As Fig. 2A shows, after the addition of 5 μM DMS in a

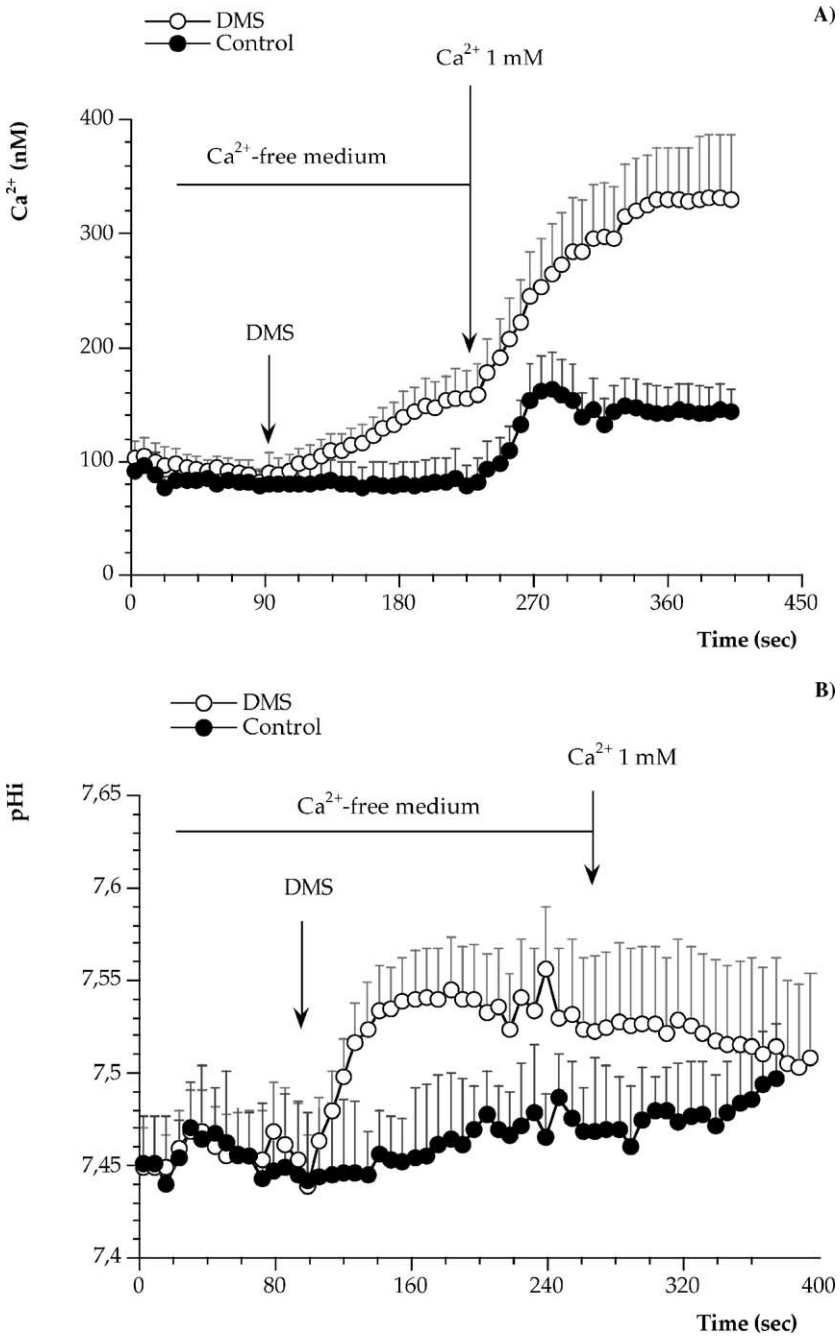

Fig. 2. Effect of DMS in cytosolic Ca^{2+} (A) and pHi (B) levels on human T lymphocytes. Ca^{2+} was removed from the extracellular medium to check intracellular Ca^{2+}-pools contribution to cytosolic calcium increase induced by DMS. The arrow indicates the addition of 5 μM DMS. One mM Ca^{2+} is again added after calcium release from the pools to restore external calcium conditions. The data are the average of 530 cells (control) and 567 cells (DMS). Mean ± SEM of three experiments. Significant differences: Ca^{2+} data: after 150 s. DMS data show significant differences with respect to the control ($P \leq 0.05$). pHi data: between 100 and 220 s DMS data show significant differences with respect to the control ($P \leq 0.05$).

Ca^{2+}-free medium, a slow release of calcium from intracellular pools takes place, while control cells show no cytosolic calcium increase; the addition of external calcium induces a significant calcium-influx. As Fig. 2B shows the release of calcium from intracellular pools is associated with a fast and significant increase in pHi, while the addition of calcium does not modify pHi. To know which intracellular calcium pools are implicated in the initial Ca^{2+}-peak we checked DMS effect in Tg-treated cells. Tg is a tumor-promoting sesquiterpene lactone that inhibits Ca^{2+}-ATPase from intracellular pools [28] and as a consequence it induces calcium pool emptying. As Fig. 3A shows, 1 μM thapsigargin activates, in the absence of external calcium, the release of calcium from intracellular pools and the influx of external calcium upon addition of this cation to the medium. After thapsigargin treatment, DMS induces an additional small depletion of intracellular calcium pools. When calcium is again added, the influx is lightly and non-significantly higher than the one induced by thapsigargin alone. In these conditions, the pHi was

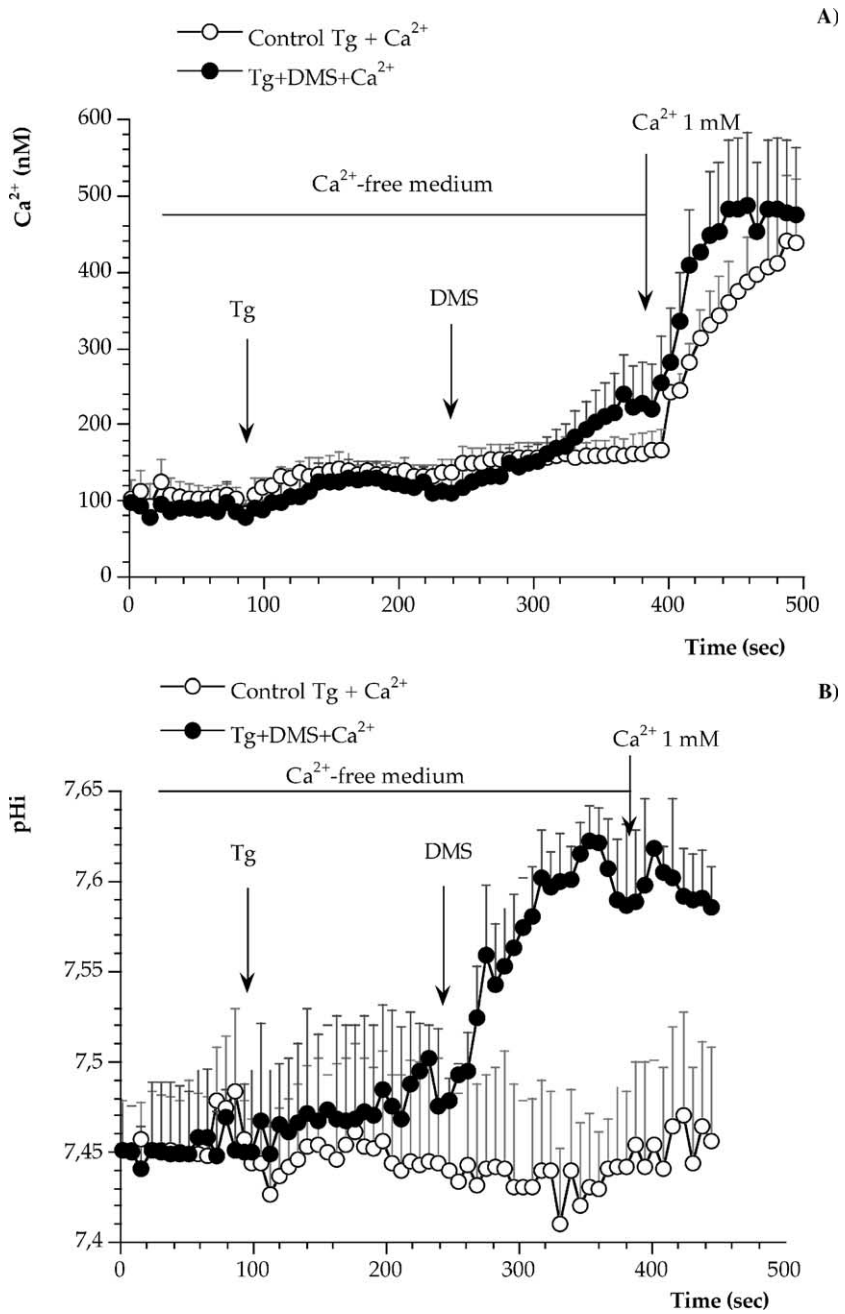

Fig. 3. Effect of thapsigargin on the increase of cytosolic Ca^{2+} (A) and pHi (B) induced by DMS in human T lymphocytes. The cells were treated for 2 min with 1 μM thapsigargin to empty the pools and then with 5 μM DMS in a Ca^{2+}-free medium (closed circles) or thapsigargin control, without DMS (open circles). One mM Ca^{2+} is again added after calcium release from the pools to restore external calcium conditions. The data are the average of 570 cells (Th control) and 625 cells (Th + DMS). Mean ± SEM of three experiments. Significant differences: pHi data: after 220 s DMS data show significant differences with respect to the control ($P \leq 0.05$).

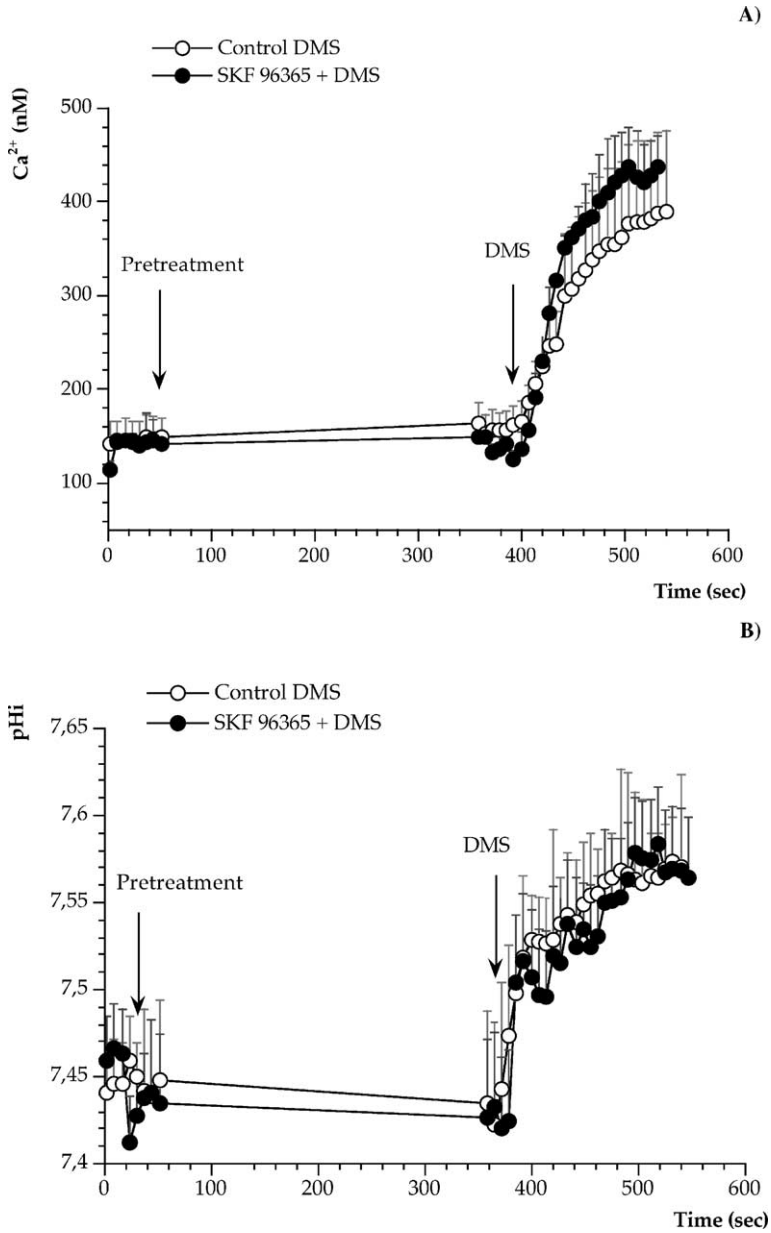

Fig. 4. Effect of SKF 96365 on the increase of cytosolic Ca^{2+} (A) and pHi (B) induced by DMS in human T lymphocytes. The cells were preincubated in the presence of 30 μM SKF 96365 (closed circles) for 5 min (pretreatment), and then incubated with 5 μM DMS. Control of DMS response (open circles). The data are the average of 585 cells (DMS control) and 435 cells (SKF 96365). Mean ± SEM of three experiments.

checked. As Fig. 3B shows thapsigargin does not induce any modification on pHi, and it does not modify the pHi increase induced by DMS either. SKF 96365 is an imidazole derivative known as blocker of Ca^{2+}-influx through SOC [14,29], the channels usually activated as a consequence of calcium pools depletion [30]. In the presence of this drug (Fig. 4), either the influx of calcium (Fig. 4A) or the increase in pHi (Fig. 4B) induced by DMS were not modified.

Then we checked the participation of other calcium channels in DMS-induced Ca^{2+}-influx. As Fig. 5 shows, the influx of calcium induced by DMS is completely blocked by preincubation in the presence of 1 mM nickel (Fig. 5B) or 1, 10 and 100 μM lanthanum (Fig. 5A), two known inhibitors of Ca^{2+} channels [31]. However, 10 and 100 μM cadmium, a non-selective inhibitor of Ca^{2+}-permeant channels [32], reduces but does not block DMS-induced Ca^{2+}-influx (Fig. 5C). In the presence of these ions, no modifications on pHi increase induced by DMS were observed (Fig. 5D–F).

In some cellular models, calcium signal is described as G-protein-dependent and pertussis toxin sensitive [33–35]. As Fig. 6A and B show, in DMS experiments, the inhibition of G-protein by 100 ng/mL pertussis toxin incubation [36] significantly reduces either calcium-influx and pHi increase stimulated by the drug. In these conditions, we checked the effect of pertussis toxin on calcium release from intracellular pools and calcium-influx as described in Fig. 3. As Fig. 7 shows, the preincubation in the presence of

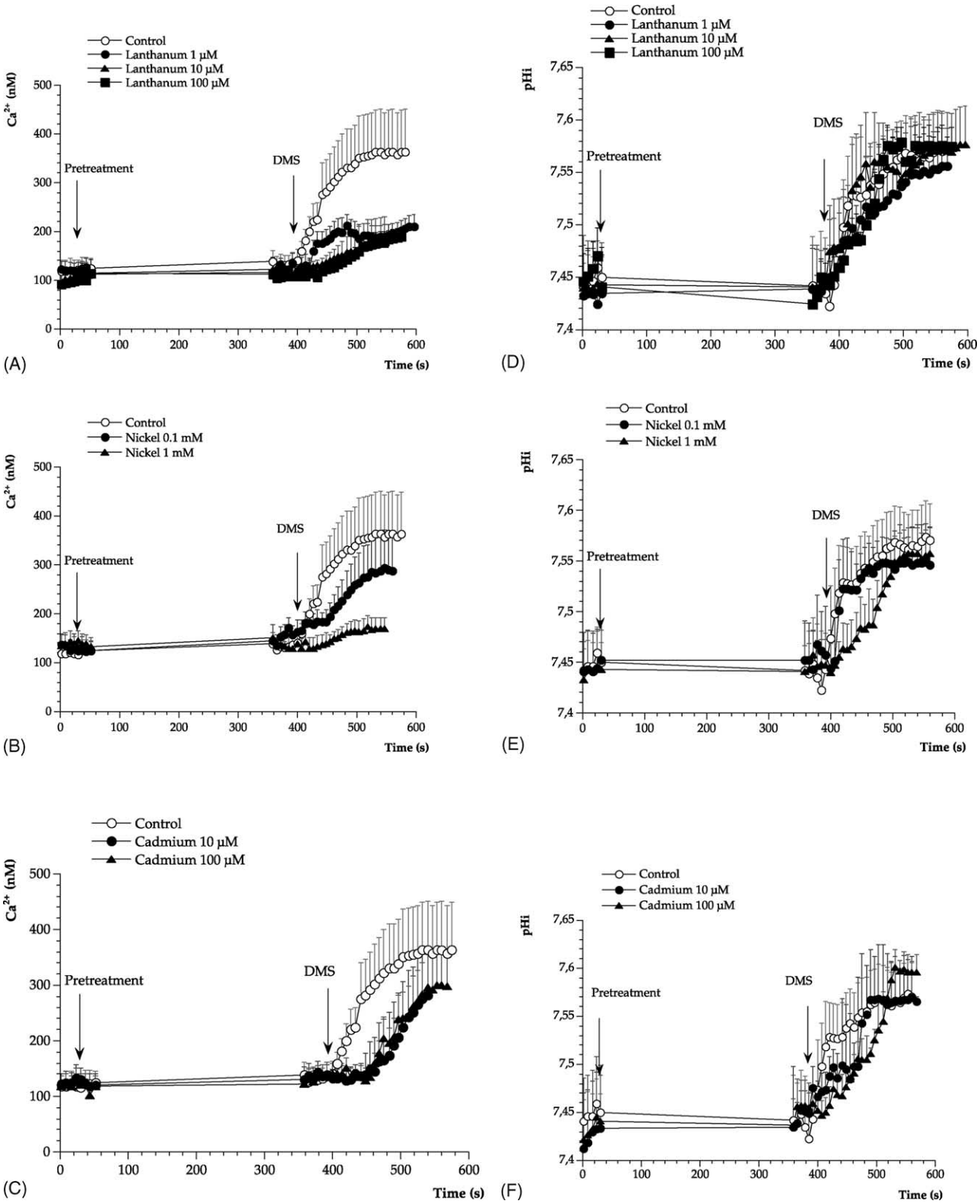

Fig. 5. Effect of calcium channel blockers on the increase of cytosolic Ca^{2+} (A–C) and pHi (D–F) induced by DMS in human T lymphocytes. The cells were preincubated in the presence of different concentrations of lanthanum (A and D), nickel (B and E), and cadmium (C and F) for 5 min (pretreatments), and then incubated with 5 μM DMS. Control of DMS response (open circles). The data are the average of 585 cells (DMS control), 410 cells (1 μM La), 440 cells (10 μM La), 500 cells (100 μM La), 587 cells (0.1 mM Ni), 605 cells (1 mM Ni), 438 cells (10 μM Cd) and 604 cells (100 μM Cd). Mean ± SEM of three experiments. Significant differences: Ca^{2+} data: after 400 s lanthanum data are significantly different with respect to the control of DMS in the three concentrations studied ($P \leq 0.05$); after 400 s 1 mM nickel data are significantly different with respect to the control of DMS ($P \leq 0.05$); between 400 and 460 s cadmium data are significantly different with respect to the control of DMS in the two concentrations studied ($P \leq 0.05$).

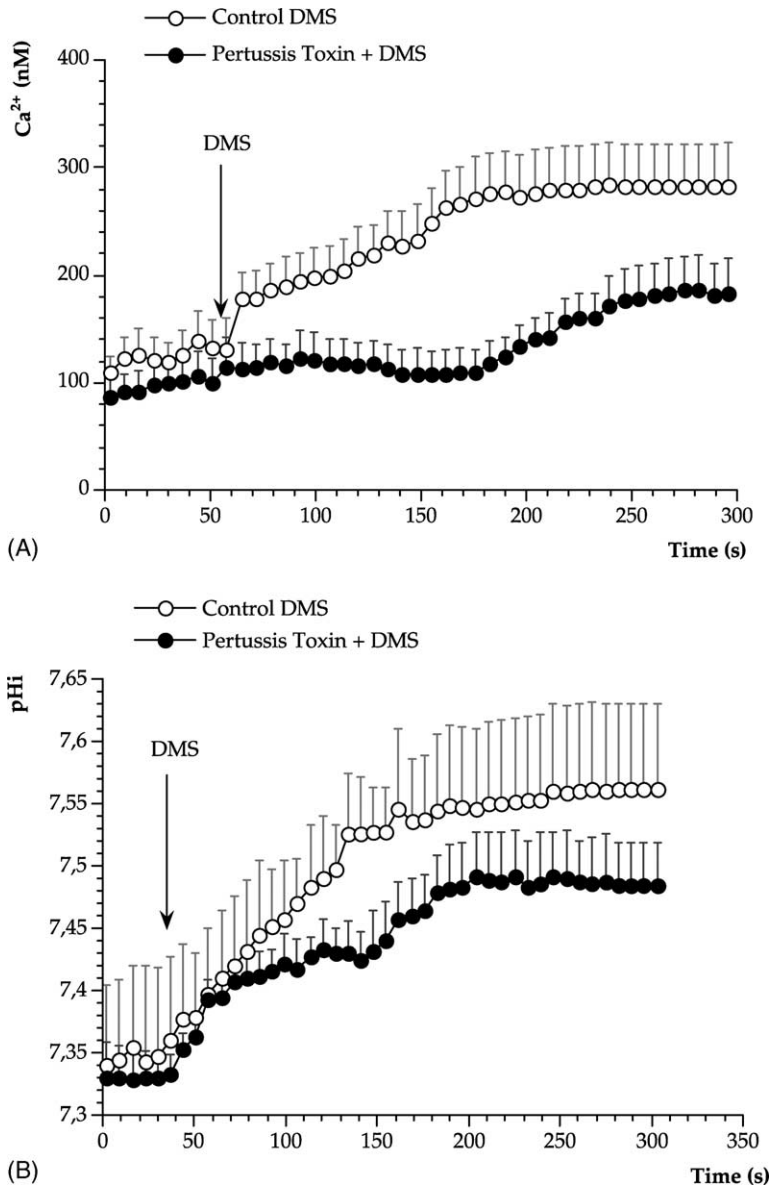

Fig. 6. Effect of pertussis toxin on the increase of cytosolic Ca^{2+} (A) and pHi (B) induced by DMS in human T lymphocytes. The cells were preincubated in the presence of 100 ng/mL pertussis toxin for 180 min (closed circles) and then incubated with 5 μM DMS. Control of DMS response (open circles). The data are the average of 625 cells (DMS control) and 735 cells (pertussis toxin). Mean ± SEM of three experiments. Significant differences: Ca^{2+} data: after 50 s DMS control data show significant differences with respect to pertussis toxin pretreatment ($P \leq 0.05$). pHi data: between 100 and 150 s DMS control data show significant differences with respect to pertussis toxin pretreatment ($P \leq 0.05$).

pertussis toxin significantly inhibits both calcium release and calcium-influx induced by DMS (Fig. 7A). However, pertussis toxin preicubation does not affect cytosolic calcium increase induced by thapsigargin (Fig. 7B). The toxin (Fig. 7C) significantly inhibits the initial pH increase induced by DMS, even after calcium addition the pHi was increased. In the presence of pertussis toxin and thapsigargin no modifications on pHi were observed (Fig. 7D).

Since DMS was described as a tyrosine kinase activator [2], we checked the effect on Ca^{2+}-influx and pHi when this pathway was inhibited. Fig. 8 shows pH and cytosolic Ca^{2+} when the tyrosine kinase pathway was inhibited in the presence of genistein [37]. Either 10 or 100 μM genistein significantly reduces intracellular calcium increase induced by DMS (Fig. 8A), however, genistein does not modify the increase in pHi observed in the presence of DMS (Fig. 8B). As Fig. 9A shows, in a Ca^{2+}-free medium, genistein significantly inhibits both the release of calcium from intracellular pools and the entry from extracellular medium induced by DMS. In these conditions, the increase of pHi induced by DMS was significantly delayed (Fig. 9B).

4. Discussion

DMS is the *N*-methyl derivative of sphingosine, both were first described as PKC inhibitors, although subsequent studies have shown different effects, as the strong

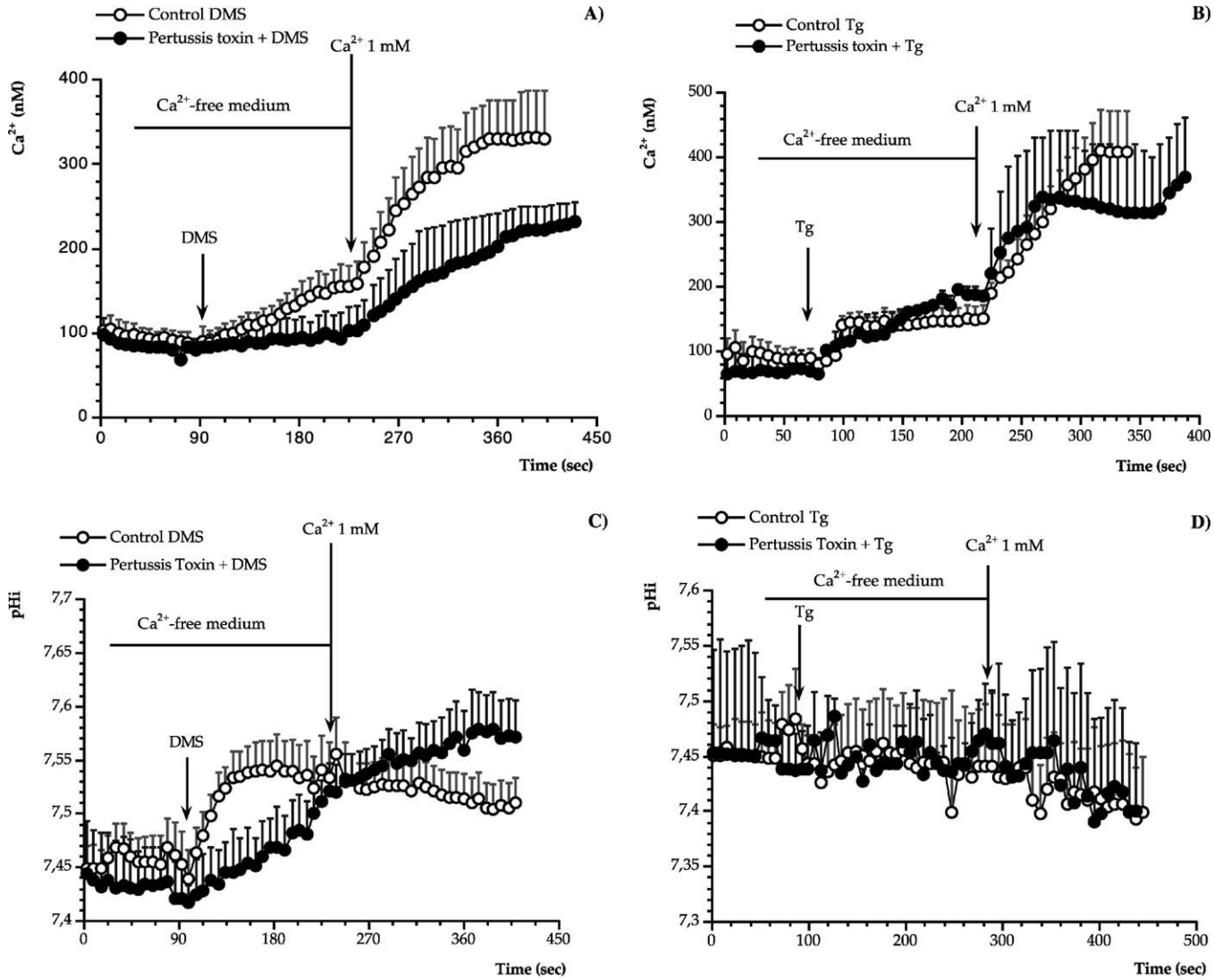

Fig. 7. Effect of pertussis toxin on the modifications on cytosolic Ca^{2+} (A and B) and pHi (C and D) induced by DMS or thapsigargin in human T lymphocytes in a calcium-free medium. (A and C) The cells were preincubated in the presence of 100 ng/mL pertussis toxin for 180 min in saline solution (closed circles) and then incubated with 5 μM DMS. Control of DMS response (open circles). One mM Ca^{2+} is again added after calcium release from the pools to restore external calcium conditions. The data are the average of 525 cells (DMS control) and 605 cells (pertussis toxin). (B and D) The cells were preincubated in the presence of 100 ng/mL pertussis toxin for 180 min in saline solution (closed circles) and then incubated with 1 μM thapsigargin. Control of thapsigargin response (open circles). One mM Ca^{2+} is again added after calcium release from the pools to restore external calcium conditions. The data are the average of 600 cells (Th control) and 625 cells (pertussis toxin). Mean ± SEM of three experiments. Significant differences: Ca^{2+} data: after 180 s DMS control data show significant differences with respect to pertussis toxin pretreatment ($P \leq 0.05$). pHi data: between 100 and 210 s DMS control data show significant differences with respect to pertussis toxin pretreatment ($P \leq 0.05$).

increase in kinase activity of epidermal growth factor receptor [2], Src kinase activity [38], sphingosine-dependent protein kinase activation [10] and calcium mobilization and influx in HL-60 cells [14].

In a previous paper, we described an increase in cytosolic Ca^{2+}-levels after DMS addition to human T lymphocytes [25], in the present paper, we characterized this increase and we study the effect on pHi. DMS induces increases in cytosolic calcium and pH levels in human T lymphocytes, these effects are dose-dependent. The calcium increase is due to the release from intracellular Ca^{2+}-pools and to the entry from the extracellular medium. It is well known relationship between the filling state of intracellular Ca^{2+}-pools and calcium-influx. In this sense, Ca^{2+}-pools depletion activates calcium-influx through SOC [30]. In our results, the empty of thapsigargin-dependent pools does not inhibit DMS effect, since the addition of DMS after thapsigargin induces a new release of calcium from intracellular pools. This new release could indicate that 1 μM thapsigargin is not enough to empty the pools and DMS is able to induce a more effective emptiness in the same pools. However, three events indicate that the pools emptied are probably different: first 2 μM thasigargin does not induce a higher release (data not show), second the addition of DMS does not induce higher calcium-influx, and finally SKF 96365, a known blocker of

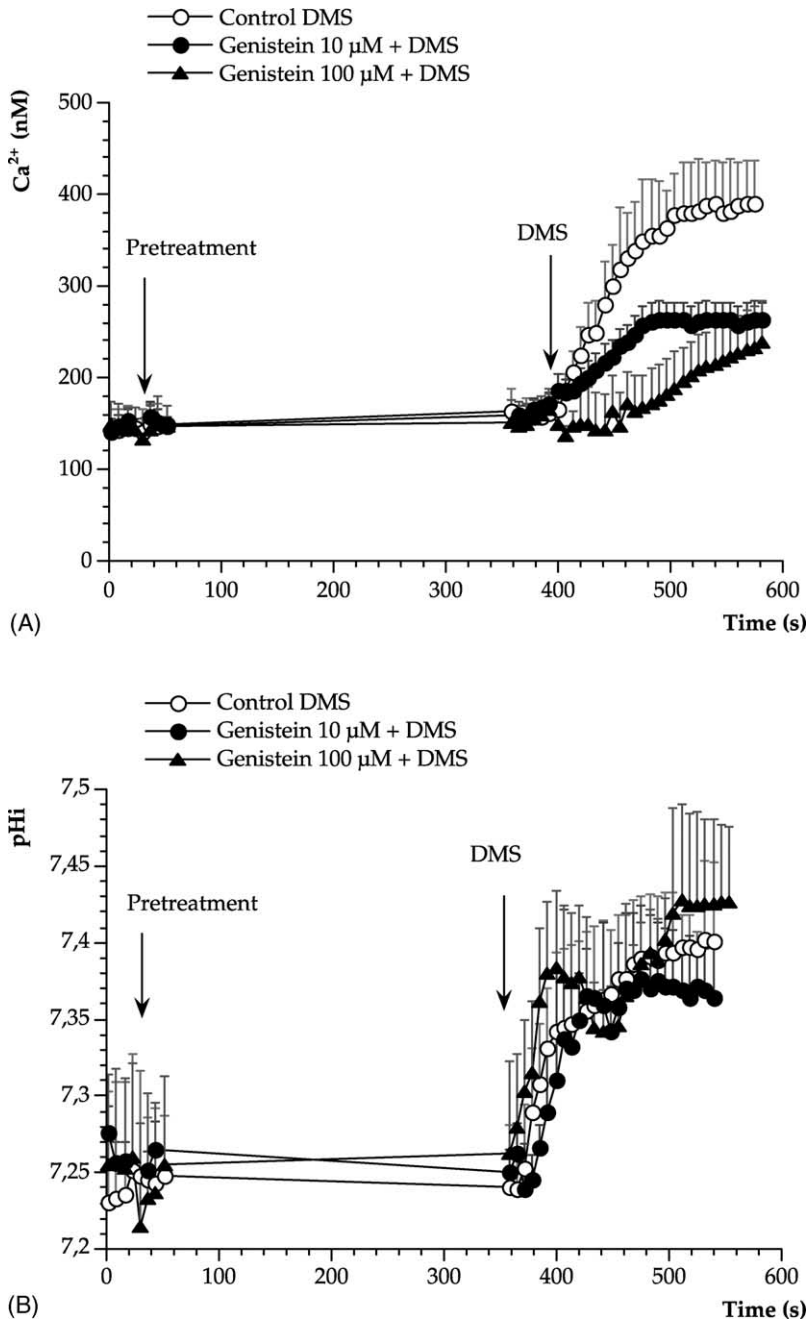

Fig. 8. Effect of tyrosine kinase modulation on the increase of cytosolic Ca^{2+} (A) and pHi (B) induced by DMS in human T lymphocytes. The cells were preincubated in the presence of 10 µM (closed circles) or 100 µM (closed triangles) genistein for 5 min (pretreatment), and then incubated with 5 µM DMS. Control of DMS response (open circles). The data are the average of 885 cells (DMS control) and 725 cells (genistein). Mean ± SEM of six experiments. Significant differences: Ca^{2+} data: after 400 s DMS control data show significant differences with respect to genistein pretreatment ($P \leq 0.05$).

Ca^{2+}-influx through SOC, that inhibits calcium-influx induced by thapsigargin [39,40], does not affect to DMS effect. Besides, it has been described that the calcium-influx induced by store depletion is blocked by cadmium [41], however, in the presence of cadmium, we only have a small inhibition of the calcium-influx induced by DMS, while thapsigargin calcium-influx is inhibited [42]. Therefore, these results point that DMS releases calcium from different pools than thapsigargin and it induces Ca^{2+}-influx not through SOC activation. In this sense, in HL-60 cells after DMS treatment it has been described both, release of Ca^{2+} from intracellular pools and an entry through different channels than SOC [14]. Although it has been also suggested that DMS can activate Ca^{2+} extrusion [43], or an inhibition in SOC [1,14].

The increase in cytosolic pH induced by DMS is associated with the release of calcium from intracellular pools, even the profile of pH increase is faster than the release of calcium from pools, which could indicate that pHi increase happens before and it is necessary to empty the pools. The

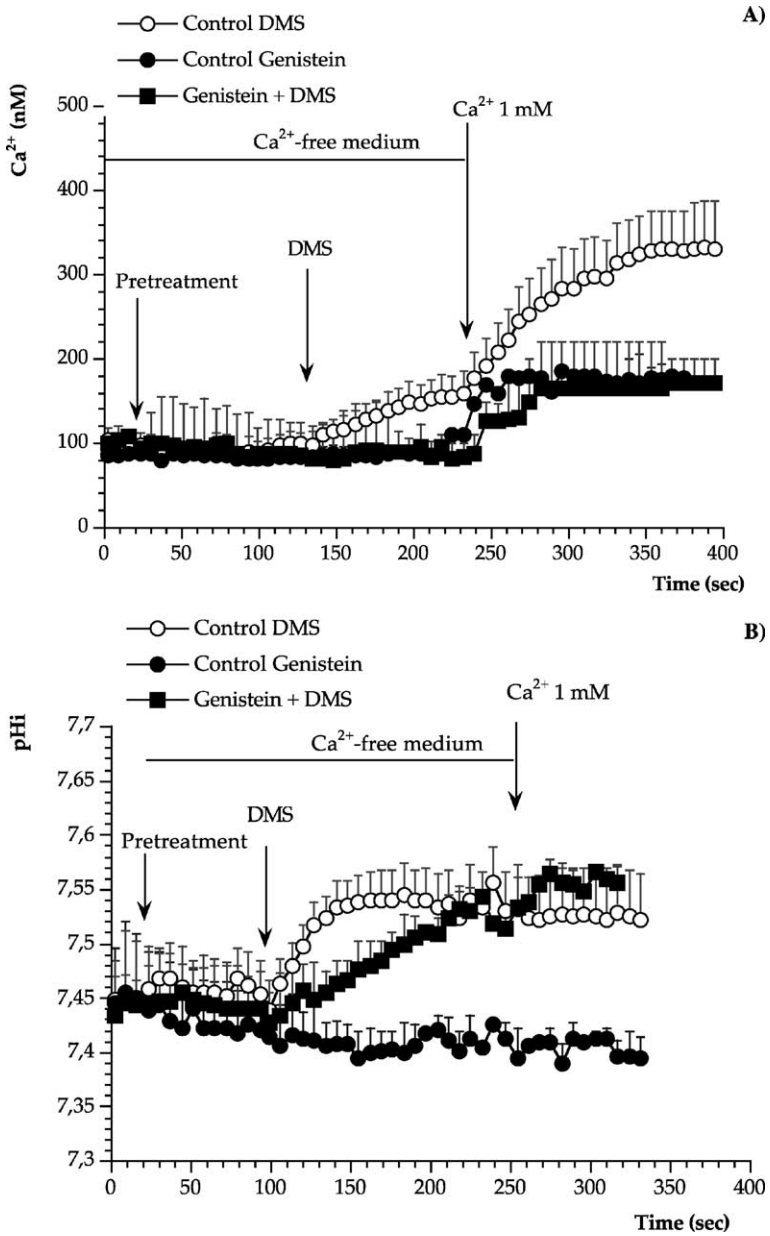

Fig. 9. Effect of tyrosine kinase modulation on the increase of cytosolic Ca^{2+} (A) and pHi (B) induced by DMS in a calcium-free medium in human T lymphocytes. Ca^{2+} was removed from the extracellular medium to check intracellular pools contribution to calcium increase induced by DMS. The cells were preincubated in the presence of 10 μM genistein for 5 min (pretreatment), and then incubated with 5 μM DMS (closed squares). Control of DMS response (open circles) and genistein effect (closed circles). One mM Ca^{2+} was again added after calcium release from the pools to restore external calcium conditions. The data are the average of 530 cells (DMS control), 607 cells (gensitein + DMS) and 614 cells (genistein control). Mean ± SEM of three experiments. Significant differences: Ca^{2+} data: after 180 s DMS data show significant differences with respect to genistein + DMS and gensitein control ($P \leq 0.05$). pHi: after 100 s genistein + DMS data show significant differences with respect to DMS control data ($P \leq 0.05$); after 100 s gensitein control data show significant differences with respect to DMS control data and genistein + DMS data ($P \leq 0.05$).

pHi increase is not dependent of calcium-influx from extracellular medium, since the inhibition of this influx in the presence of nickel or lanthanum does not affect to pHi increase induced by DMS. This result again point to a different effect of thapsigargin and DMS, since pHi is not modified in the presence of thapsigargin. The relationship of calcium pools, different than thapsigargin-dependent pools, and cytosolic alcalinization was described in several cellular models [44–46], even as far as we know this is the first time in describing DMS effect on cytosolic calcium through pHi.

We have been described in a previous paper, the relationship between pHi and cytosolic calcium in human lymphocytes [24]. In those conditions, the cytosolic alcalinization induced by NH_4Cl slightly decrease cytosolic calcium. That is not contradictory to present results since NH_4Cl data were done in a bicarbonate-free solution, and as happens in other cellular models, the presence of

bicarbonate ion in the extracellular medium induces different behavior in cytosolic calcium [47].

The fact that Ca^{2+}-increase induced by DMS is dependent on extracellular calcium is confirmed by results obtained with lanthanum and nickel. Both completely block the calcium-influx induced by DMS, and they do not affect to the release of calcium from internal stores, since in the presence of these two ions a small (100 nM) calcium increase happens after DMS addition, even when the blocker concentration was higher. In the case of these two ions, the influx of calcium induced by thapsigargin is also blocked since these ions are blockers of any calcium entry through non-voltage-dependent calcium channels [31,48,49].

The DMS-influx is also dependent on a pertussis-sensible G-protein, because in the presence of this toxin the influx is inhibited. Pertussis toxin results are very interesting since toxin pretreatment inhibits both the release from internal stores and the influx from extracellular medium, while it does not affect to thapsigargin effect. In addition, pertussis toxin induces an initial inhibition in pHi increase induced by DMS. These results indicate that part of DMS effect is probably due to the interaction with some membrane structure coupled to G-proteins. Some studies suggest that exogenous sphingosine 1-phophate increases cytosolic calcium *via* cell surface G-protein coupled receptors that activate phospholipase C, i.e. muscarinic receptors [35]. Since sphingosine derivatives have a similar structure, the G-protein activated by receptor and the one that modulates DMS response could be the same. But, the calcium-influx activates by exogenous sphingosine 1-phophate is capacitative and DMS seems to have a inhibitory role in this influx [50]. Therefore, the G-protein that modulates DMS effect is probably different than the one activated by sphingosine1-phosphate. Even other possibility is the same G-protein but different receptor and intracellular mediator. In any case, pertussis toxin results again point to a different mechanisms of action for DMS and thapsigargin.

DMS has been considered as a tyrosine kinase activator [2], when this pathway was inhibited we obtained an important decrease in calcium-influx induced by DMS, even an increase of 100 nM of cytosolic calcium was observed. When calcium was eliminated from the extracellular medium and tyrosine kinases were inhibited, the inhibition of calcium uptake induced by DMS was complete. In this case both, calcium release from internal pools and calcium-influx, were inhibited. The difference between both results, that is genistein in a calcium-free or a calcium-containing medium, was also observed in pH results since in a calcium-containing medium genistein does not modified DMS effect on pHi, while in a calcium-free medium it induces a delayed in the increase in pHi induced by DMS, even the final pH rich the same value than DMS control. This different behavior of genistein could be explained together with pertussis toxin results, both pertussis toxin and genistein inhibit pHi increase in a calcium-free medium and as consequence the release of calcium from intracellular pools. The final pHi, before calcium re-addition, rich the same values of DMS control but initially the cell is able to compensate the pHi increase and as consequence the emptiness of pools does not happen. However, when calcium is present, pertussis toxin only induces a small delay and genistein does not affect to pHi increase, so the release of calcium from intracellular takes place, in fact in both cases cytosolic-Ca^{2+} is increased in 100 nM. On the other hand, both drugs inhibit independently the influx of calcium. In several cellular models cytosolic alkalinization is enough signal to release calcium from intracellular pools [44,51]; however, this emptiness of calcium induced by alkalinization is not always related with a significant calcium entry [52]. In this case, the fast alcalinization induced by DMS seems to be the responsible of calcium pools emptiness, and the Ca^{2+}-influx is not related with pools state, although the three events are independently related with G-proteins and tyrosine kinases.

It has been reported elsewhere that in fibroblasts DMS activates a serine kinase called sphingosine-dependent protein kinase (SDK) different from other protein kinases that can be involved in signal transduction [10]. This sphingosine-dependent protein kinase is also activated by the DMS-precursor sphingosine, and this drug also increases calcium-influx (data not shown). The sphingosine-dependent protein kinase family phosphorylates 14-3-3 proteins and tyrosine kinases phosphorylates residues that have not been mapped [10]. Therefore, this kinase could be a candidate for DMS action and calcium effect in T lymphocytes even a connection of sphingosine-dependent protein kinase and G-proteins never has been establish.

In summary and taking together these results, DMS increases calcium-influx by opening a channel, different from SOC, modulated by a G-protein, tyrosine kinases and blocked by nickel and lanthanum. This channel is probably the same as the undefined Ca^{2+}-channel described in HL-60 cells [14]. On the other hand, DMS increases pHi and as consequence induces the release of calcium from intracellular pools. This increase is dependent of G-protein and tyrosine kinase pathway but independent of calcium-influx. This role of DMS in pH and calcium signals suggests a new way of DMS and sphingosine to modulate intracellular functions [3,10].

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