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Regulation of calcium signalling by 1-O-alkylglycerols in human Jurkat T lymphocytes

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Abstract

We studied the role of natural occurring 1-O-alkylglycerols on the calcium signalling in Jurkat T-cells. Alkylglycerols evoked an increase in free intracellular calcium concentration $[Ca^{2+}]i$, in a dose-dependent manner. When the experiments were performed in calcium-free buffer, the alkylglycerol response on the rise of $[Ca^{2+}]i$ was wholly abolished compared with the one in calcium-containing buffer, suggesting that these etherlipids induce a calcium influx by the opening of Ca^{2+} channels. We further employed inhibitors of voltage-gated calcium channels. We observed that ω-conotoxin, a blocker of N-type voltage-activated Ca^{2+} channels, but not verapamil, a blocker of L-type voltage-activated Ca^{2+} channels, curtailed significantly the calcium rise evoked by the lipid agents. Alkylglycerols also induced plasma membrane depolarisation, known to be involved in the opening of the voltage-gated calcium channels. Our study shows that alkylglycerols increase $[Ca^{2+}]i$ influx in human Jurkat T-cells possibly by modulating the permeability of calcium channels.

Keywords: 1-O-alkylglycerols; Calcium channels; Plasma membrane potential; Jurkat T-cell

Introduction

T lymphocyte activation is an essential event in immune responses during which numerous transcription factors are stimulated, resulting in the gene expression such as interleukin-2 and other lymphokines. Enhanced gene transcription is mediated by intracellular second messengers like calcium. Indeed the increase in free cytosolic calcium concentrations, $[Ca^{2+}]i$, is involved in the transcriptions of

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several genes, triggering signal for T-cell activation in immune defense. The $[Ca^{2+}]i$ dependence stems from extracellular Ca^{2+} influx or the Ca^{2+} release from intracellular stores. According to the capacitative model of calcium entry, calcium is first released after antigen receptor activation from intracellular pools, mainly endoplasmic reticulum, and then is extruded into the extracellular medium (Putney, 1990). In turn, the cells refill their intracellular emptied pool by opening calcium channels in the plasma membrane such as Ca^{2+} release-activated Ca^{2+} (CRAC) channels in Jurkat T-cells (Lewis, 2001; Fomina et al., 2000). The $[Ca^{2+}]i$ rise also may be due to the Ca^{2+} influx through voltage-gated calcium channels (VGCC) after antigen receptor activation (Densmore et al., 1992). Three groups of VGCC were described such as high, intermediate and low voltage-activated calcium channels (Moreno Davila, 1999). VGCC open in response to depolarisation of the plasma membrane, triggering then a fast Ca^{2+} influx (Ricci et al., 1996). According to Cens et al., calcium oscillations mediated by VGCC can be inhibited either by the changes in membrane potential (voltage-dependent inactivation) or by the consecutive entry of Ca^{2+} (Ca^{2+}-dependent inactivation) (Cens et al., 1999). Thus elevation of $[Ca^{2+}]i$ inducing subsequent T lymphocyte response may be regulated by external effectors through the modulation of plasma membrane channel opening.

1-O-alkylglycerols (Alkyl-Gro) are natural occurring etherlipids found in abundance in shark liver oil, and at a lesser extent, in haematopoietic organs or maternal milk (Hallgren and Larsson, 1962; Hallgren et al., 1974). Alkyl-Gro possess many biological activities such as antitumoral effects (Brohult et al., 1978), haematopoiesis stimulation (Linman, 1960) or fertility and motility improvement (Cheminade et al., 2002). Furthermore, Yamamoto et al. have previously reported that alkyl-Gro are potent macrophage-activating agents (Yamamoto and Ngwenya, 1987; Yamamoto et al., 1988) and their effects require T- and B-cells contribution (Homma et al., 1990; Yamamoto et al., 1991). However, the studies on the implication of alkyl-Gro in T-cell signalling are lacking. Keeping a view the paucity of information on the subject, it was thought worthwhile to undertake the present study to elucidate the effects of alkyl-Gro in the modulation of calcium signalling in human T-cells. Indeed, neither 1-O-octadecyl-*sn*-glycerol tested in human neutrophil granulocytes nor 1-O-hexadecyl-*sn*-glycerol experienced on Zellweger fibroblasts induced any cellular calcium oscillations (Palmblad et al., 1990; Clark and Murray, 1995). Thus we were interested to determine the potential effect of alkyl-Gro extracted from *Centrophorus squamosus* liver oil on the human Jurkat T lymphocyte activation and we have studied the implication of such etherlipids on the calcium fluxes and their mechanisms of action.

Methods

Materials

The culture medium RPMI-1640 and L-glutamine were purchased from Biowhitaker (Emerainville, France). Fura-2/AM and bis-oxonol (bis-5(1,3-diethylthiobarbiturate) trimethioxonol) were procured from Molecular Probes (Eugenes, Oregon, USA). All other chemicals including arachidonic acid (AA, 20:4n-6) were obtained from Sigma-Aldrich (La Verpillère, France). 1-O-alkylglycerols were kindly provided by Dr P. Allaume from the Centre Technique ID-Mer (Lorient, France). Alkyl-Gro were prepared from *Centrophorus squamosus* shark liver oil as described previously (Hichami et al., 1997). Alkyl-Gro species varied according to the alkyl-chain length, whose composition was as follows: 14:0 = 0.7%, 16:0 = 9.1%, 16:1n-7 = 12.5%, 18:1n-9 = 68.1%, 18:1n-7 = 4.8% and other minor species = 4.8%.

Cell culture

The human (Jurkat) T-cells were kindly provided by Dr. Bent Rubin from UMR-CNRS Research Unit at CHR (Toulouse, France). The cells were cultured in RPMI-1640 medium supplemented with L-glutamine and 10% foetal calf serum at 37 °C in a humidified chamber containing 95% air and 5% CO_2. Cell viability was assessed by trypan blue exclusion test.

Measurement of Ca^{2+} signalling

The cells (2×10^6 per ml) were washed with phosphate-buffered saline, pH 7.4 and then incubated with Fura-2/AM (1 μM) for 60 min at 37 °C in loading buffer containing NaCl (110 mM), KCl (5.4 mM), $NaHCO_3$ (25 mM), $MgCl_2$ (0.8 mM), KH_2PO_4 (0.4 mM), HEPES-Na (20 mM), $NaHPO_4$ (0.33 mM), $CaCl_2$ (1.2 mM), which pH was adjusted at 7.4. After loading, the cells were washed three times (2000 rpm, 10 min) and remained suspended in the buffer. For experiments in Ca^{2+}-free medium, $CaCl_2$ was replaced by EGTA (1 mM) just before the time of stimulation. The intracellular concentration of free calcium $[Ca^{2+}]i$ was measured according to the Grynkiewicz's method (Grynkiewicz et al., 1985). The fluorescence intensities were monitored in the cuvette at 37 °C and determined by the ratio mode in PTI spectrofluorometer at 340 nm and 380 nm (excitation filters) and at 510 nm (emission filter). The cells were continuously stirred throughout the experiment and the test molecules were added into the cuvette. The intracellular concentrations of free Ca^{2+} were calculated by using the following formula: $[Ca^{2+}]i = Kd.(F - Fmin)/(Fmax - F)$. A value of 224 for Kd was applied to the calculations. Fmax and Fmin values were obtained by addition of ionomycin (5 μM) and $MnCl_2$ (2 mM), Triton-X100 (0.1%), EGTA (24 mM), respectively. The calcium oscillation ($\Delta[Ca^{2+}]i$) triggered by the test molecule addition was evaluated by the difference of $[Ca^{2+}]i$ present in unstimulated cells and in test molecule-stimulated cells.

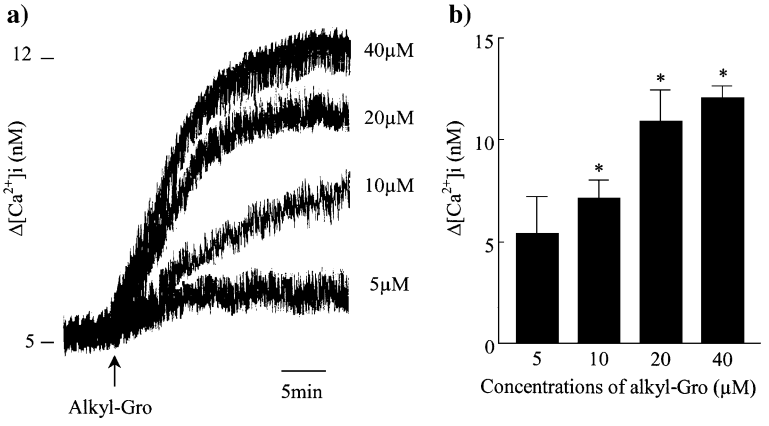

Fig. 1. Alkyl-Gro effect on the $[Ca^{2+}]i$ rise in Jurkat T-cells. Cells (4×10^6 per assay) were loaded with the fluorescent probe Fura-2/AM and the test molecules were added into the cuvette as indicated by the arrowheads. Upward deflections correspond to the rise of $[Ca^{2+}]i$ in these cells. **a)** The figure shows the single traces of observations, which were reproduced at least three times independently. **b)** $n \geq 3 \pm$ SEM. Values were compared to the control using Student's *t*-test of significance: *, $p < 0.05$.

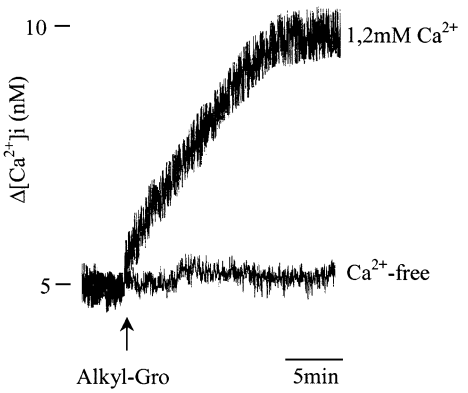

Fig. 2. Effect of extracellular calcium on alkyl-Gro-evoked increase in $[Ca^{2+}]i$ in Jurkat T-cells. Cells (4×10^6 per assay) were loaded with the fluorescent probe Fura-2/AM. Alkyl-Gro (20 μM) were added into the cuvette containing cells suspended into the calcium buffer (1.2 mM Ca^{2+}) or suspended into the calcium-free buffer (Ca^{2+}-free, in the latter Ca^{2+} was replaced by EGTA). The figure shows the single traces of observations, which were reproduced three times independently.

Measurement of membrane potential, Vm

The cells were prepared as described for $[Ca^{2+}]i$ determinations. After washing, the cells (2×10^6 per ml) were transferred to the spectrofluorometer cuvette where bis-oxonol (150 nM) was added (Rink et al., 1980). The cells were allowed to equilibrate with the dye and, after 10 min, different test molecules were added. The fluorescence intensities were determined at 540 nm (excitation filter) and at 580 nm (emission filter). Downward and upward deflections represent hyper- and de-polarisation, respectively.

Statistical evaluation

Data are presented as the mean ± SEM of the indicated number of experiments; each performed separately at least three times. The significance of the difference between each treatment was tested by individual paired *t*-test.

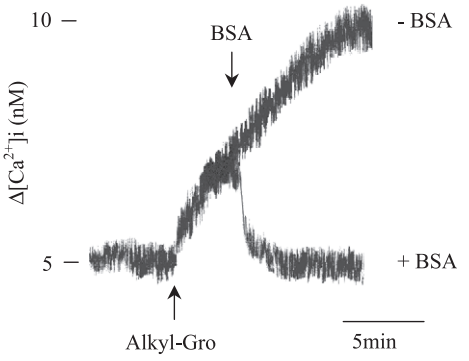

Fig. 3. Effect of BSA addition on alkyl-Gro-evoked rise in $[Ca^{2+}]i$ in Jurkat T-cells. Cells (4×10^6 per assay) were loaded with the fluorescent probe Fura-2/AM. Alkyl-Gro (20 μM) and then BSA (0.2%, w/v) were added to the cuvette as indicated by the arrows. The figure shows the single traces of experiments, which were reproduced three times independently.

Results

Alkyl-Gro evoke a [Ca^{2+}]i rise in Jurkat T-cells

Alkyl-Gro induced an increase of cytosolic calcium rate in Jurkat T-cells as a function of time, which reached a near plateau after 20 minutes (Fig. 1a). Alkyl-Gro evoked a rise in [Ca^{2+}]i in a dose-dependent manner (Fig. 1b).

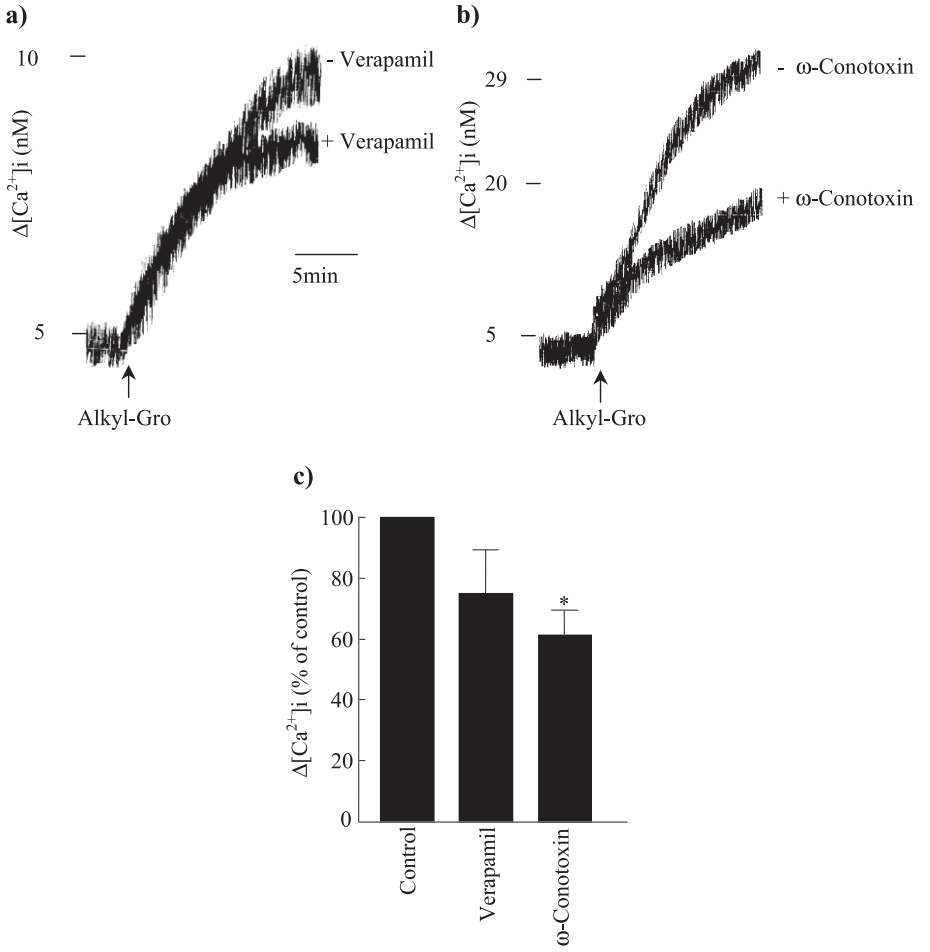

Fig. 4. Effect of verapamil and ω-conotoxin on the alkyl-Gro-evoked rise in [Ca^{2+}]i in Jurkat T-cells. Cells (4.10^6 per assay) were loaded with the fluorescent probe Fura-2/AM. The cells were preincubated in the presence of verapamil (1 μM) and ω-conotoxin (1 μM) for 15 min. Alkyl-Gro (20 μM) were then added to the cuvette as indicated by the arrowheads. a and b show the single traces of observations, which were reproduced independently. c represents the means of at least three experiments. Values ± SEM were compared to the control using Student's *t*-test of significance: *, $p < 0.05$.

Alkyl-Gro induce a calcium influx in Jurkat T-cells

We conducted experiments in the absence (Ca^{2+}-free) and in the presence (1.2 mM Ca^{2+}) of calcium in the buffer. The increases in [Ca^{2+}]i evoked by alkyl-Gro in 1.2 mM Ca^{2+}-buffer were completely abolished when experiments were performed in Ca^{2+}-free buffer (Fig. 2).

In order to assess whether alkyl-Gro act extracellularly in calcium signalling, we used the fatty acid-free BSA (0.2% (w/v)) which compete at this concentration with lipids bound to the plasma membrane. Addition of BSA after alkyl-Gro abruptly abolished the rise in [Ca^{2+}]i induced by the latter in Jurkat T-cells (Fig. 3).

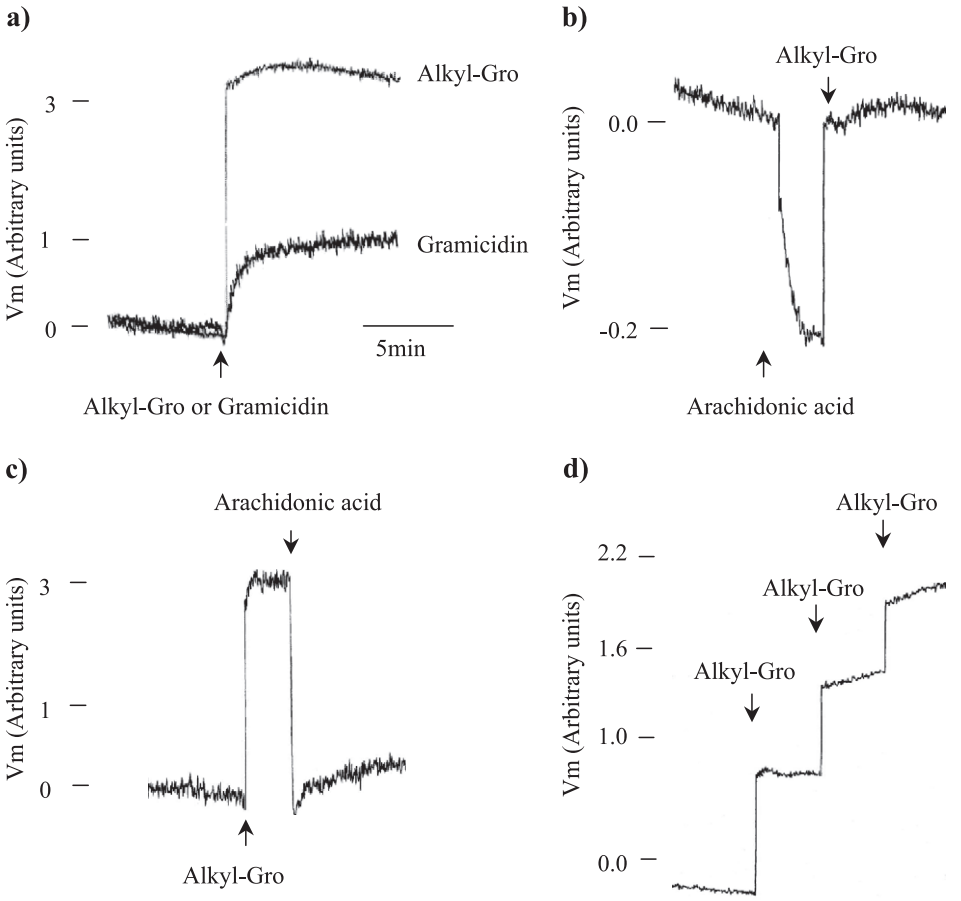

Fig. 5. Effect of alkyl-Gro, gramicidin and arachidonic acid on membrane potential (Vm) in Jurkat T-cells. Cells (4×10^6 per assay) were loaded with the fluorescent probe bis-oxonol. The arrowheads indicate the time when the test molecules, alkyl-Gro (20 μM), gramicidin (1 μM) and arachidonic acid (10 μM), were added into the cuvette. The figure shows the single traces of experiments, which were reproduced three times independently.

Effects of Ca^{2+}-channel blockers on alkyl-Gro-enhanced rise in $[Ca^{2+}]i$

In order to explore the mechanisms by which alkyl-Gro brings about an increase of $[Ca^{2+}]i$, we used Ca^{2+}-channel blockers. Hence, verapamil, an inhibitor of L-type VGCC, did not affect the $[Ca^{2+}]i$ rise stimulated by alkyl-Gro (Fig. 4a). Nevertheless, ω-conotoxin, known to block N-type VGCC, abolished partially the alkyl-Gro-activated entry of extracellular Ca^{2+}, up to 39% of the maximal calcium rise (Fig. 4b,c).

Alkyl-Gro modulate membrane potential by inducing a depolarisation in Jurkat T-cells

In order to probe whether alkyl-Gro create membrane perturbations, we assessed their effect on the modifications in membrane potential (Vm). As experimental controls, we employed gramicidin, known to induce depolarisation, and AA shown to provoke hyperpolarisation. We observed that alkyl-Gro evoked plasma membrane depolarisation 3 times higher than gramicidin (Fig. 5a). Addition of AA evoked hyperpolarisation in these cells (Fig. 5b). Addition of AA after alkyl-Gro reversed the depolarisation induced by the latter (Fig. 5c). Moreover, successive addition of alkyl-Gro induced additive increases in plasma membrane depolarisation in Jurkat T-cells (Fig. 5d).

Discussion

Inflamed cancerous tissues produce alkyl-Gro, which result from the catabolism of etherlipids in tumour cells (Snyder and Wood, 1969). It has been reported that alkyl-Gro are potent modulators of the functions of macrophages, B- and T-cells (Yamamoto et al., 1988). The modulation of B-cell functions by alkyl-Gro requires induction in T-cell activation (Homma and Yamamoto, 1990). The mechanism of action of alkyl-Gro on T-cell stimulation remains particularly unclear. In the present study, we investigated the effect of alkyl-Gro on calcium signalling in Jurkat T-cells. Therefore naturally occurring alkyl-Gro evoked a slow and concentration-dependent rise in $[Ca^{2+}]i$ followed by a near plateau. However, alkyl-Gro do act extracellularly, as addition of BSA abolished the Ca^{2+} rise evoked by the formers. Hence, BSA seems to detach the plasma membrane bound-alkyl-Gro that entails the recovering to the resting level of Ca^{2+} concentration.

We were further interested in assessing whether these agents mobilize calcium from intra- or extracellular pools. We conducted experiments in calcium containing and calcium-free buffers. We observed that alkyl-Gro failed to induce increases in $[Ca^{2+}]i$ in the absence of extracellular calcium. These observations suggest that alkyl-Gro-enhanced increases in $[Ca^{2+}]i$ are due to calcium influx. Thus, alkyl-Gro did not induce the calcium release from endoplasmic or mitochondria Ca^{2+} stores. Calcium influx mainly results from the opening of CRAC channels in the plasma membrane in human Jurkat T-cell that leads to lymphocyte activation (Lewis and Cahalan, 1995). These store-operated Ca^{2+} channels, described as the capacitative model (Putney, 1990) are highly selective for calcium ions and sustain the oscillatory intracellular calcium concentration signal required for gene expression in lymphocytes (Lewis, 2001).

We were also interested in VGCC, which serve as one of the important mechanism for fast calcium flux into the cells (Moreno Davila, 1999). A voltage-operable current involved in calcium entry has been described in lymphocytes (Densmore et al., 1996), however the existence of VGCC in lymphocytes is

not widely accepted (Lewis and Cahalan, 1995). We therefore tested verapamil, a papaverine derivative inhibitor of L-type VGCC (Striessnig et al., 1998). The preincubation of T-cells with this agent did not affect significantly the alkyl-Gro-induced $[Ca^{2+}]$i rise. On the contrary, when ω-conotoxin, a blocker of N-type VGCC (Lalo et al., 2001), was applied, the $[Ca^{2+}]$i rise induced by alkyl-Gro was significantly lowered. This observation suggests that alkyl-Gro might enhance Ca^{2+} influx in part via opening the voltage-dependent N-type calcium channels. These results were strengthened by our further observations on the modulation of membrane potential. Indeed, alkyl-Gro induced a high and fast depolarisation of plasma membrane, greater than even gramicidin in Jurkat T-cells.

It is enticing to relate alkyl-Gro-induced depolarisation and $[Ca^{2+}]$i rise. However, alkyl-Gro-induced $[Ca^{2+}]$i rise was only partially abolished by the N-type VGCC blocker, suggesting other mechanisms. Alkyl-Gro activities may have several targets in the field of ion channels and permeability, and will deserve further studies.

This work is not in agreement with previous results. Indeed Clark et al. demonstrated that bradykinin-induced rise in Ca^{2+} concentration was unaffected by supplementation of human Zellweger fibroblasts with 1-O-hexadecyl-*sn*-glycerol (Clark and Murray, 1995). Nevertheless, etherlipids were preincubated for 24 h and not directly tested on the calcium oscillations. Besides, Palmblad et al. also established that 1-O-octadecyl-*sn*-glycerol did not enhance the calcium influx in human neutrophil granulocytes (Palmblad et al., 1990). According to the authors, the phosphatidyl group in *sn*-3 position may be required for calcium responses. We proved in our cell model that the simple structure of alkyl-Gro is sufficient to induce the calcium influx. The cell type could therefore influence the alkyl-Gro response implying Ca^{2+} signalling. Moreover, these two works were performed with pure alkyl-Gro species whereas this present one tested a mixture of alkyl-Gro extracted from shark liver oil, mainly composed of 1-O-octadecen(Δ9)yl-*sn*-glycerol. Further investigations may be required to clear up the mechanism of action of alkyl-Gro in Jurkat T-cells. It would be thus of interest to determine the importance of the alkyl-Gro structure, and notably the impact of the Δ9 ethylenic bond of the aliphatic alkyl-chain.

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