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An Absence of Non-specific Immune Response Towards *para*-Sulphonato-calix[*n*]arenes

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Abstract

The effects of *para*-Sulphonato-calix[4]arene, *para*-Sulphonato-calix[6]arene and *para*-Sulphonato-calix[8]arene on the activation of NADPH oxidase in neutrophils has been studied. All three molecules do not induce NADPH oxidase activation, and hence do not stimulate neutrophils. Measurement of cell viability demonstrates that these three water-soluble calix[*n*]arene derivatives are not cytotoxic.

Introduction

Recently, the applications of the calix[*n*]arenes in biological and biopharmaceutical applications have seen rapid development [1]. Their use, as passive systems for drug transport, including nanocapsules [2], solid lipid nanoparticles [3] or as simple inclusion systems for bioactive molecules [4] and as intrinsic bioactive molecules, including application as enzyme or ion channel inhibitors [5, 6], antiviral [7], antithrombotic [8, 9], synthetic antigens [10], nucleotide complexing agents [11], demonstrates the versatility of these synthetic macrocycles, derived from phenol and formaldehyde [12]. Of the wide range of available calix[*n*]arene derivatives, the *para*-Sulphonato-calix[*n*]arenes show high aqueous solubility and may be amongst the most interesting of the derivatives for biomedical applications. Indeed, recently the first report on the use of *para*-Sulphonato-calix[*n*]arenes as drug carriers, for nifedipine, was reported by Yang *et al.* [13].

Given the building blocks used in their synthesis, it would, at first, seem surprising that as yet no toxic effects have been observed for these molecules. Work by Reinhoudt [14], showed a lack of immune response to calix[4]arene coupled to serum albumins. More recently, we have shown that neither solid lipid nanoparticles [15] nor *para*-Sulphonato-calix[*n*]arenes [16], show haemolytic effects. In order to extend our knowledge of the, as yet lack, of toxicological potential of the calix[*n*]arenes, non-specific immune response is a logical target.

Polymorphonuclear neutrophils (PMNs) represent approximately 50% of blood leukocytes. Neutrophils are phagocytic cells that are rapidly recruited from the blood stream to the infection site where they play a crucial role in the early response of the organism against invading pathogens. This defence process involves phagocytosis of the bacterial agent and release of toxic radicals like superoxide anions. *In vivo*, superoxide anions are generated by the NADPH oxidase (EC 1.6.99.6), a multi-component complex [17, 18]. The catalytic core of the complex is the membrane cytochrome b_{558}. It is a heterodimer formed by a large glycosylated subunit, gp91-phox and a small subunit, p22-phox [19]. This enzyme needs activation to catalyze the NADPH-dependent reduction of O_2 into the superoxide anion $O_2^{\cdot -}$. In the activation process, cytosolic proteins, p67-phox, p47-phox, p40-phox and the monomeric G protein Rac, are recruited to the membrane cytochrome b_{558} [20, 21]. The association of the cytosolic constituents induces the conformational change of cytochrome b_{558} that leads to the activation of the electron transfer [22]. *In vivo*, the phagocyte NADPH oxidase activation can be mediated by soluble (chemotactic factors) or particulate (bacteria or fungi) stimuli, and also by an amphiphilic agent, the phorbol myristate acetate (PMA) [17]. In neutrophils, activation of the NADPH oxidase complex is a good tool to assess the stimulation-degree of the cells. In the present study,

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we have evaluated the effect of the *para*-Sulphonato-calix[*n*]arenes on NADPH oxidase activation of human neutrophils.

Material and methods

Abbreviations

PBS, phosphate-buffered saline (2.7 mM KCl, 136.7 mM NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4); PMA, phorbol myristate acetate; PMN, polymorphonuclear neutrophil; RLU, relative luminescence unit.

Material

Chemical reagents used in this study were obtained from the following sources: PMA, Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) (Sigma). Annexin-V-FITC solution and propidium iodide (PI) were obtained from Roche.

Calixarenes synthesis and solubilisation

para-H-calix[4]arene and *para*-Sulphonato-calix[*n*]arenes were prepared by literature methods [23, 24]. Their physical data is in full accord with literature values.

A stock solution of 10 mM calixarenes was prepared in PBS. The pH of the solution was adjusted to pH 7.3 with NaOH.

Purification of human neutrophils

Human neutrophils were isolated from donor blood stored overnight at 4 °C as previously described [25]. Briefly, buffy coats were diluted in PBS containing 13 U/ml heparin, and centrifuged through Ficoll/sodium metrizoate (Ficoll–Paque) for 30 min at 500×*g* at 20 °C. Neutrophils were isolated from the cell pellets after hypotonic lysis of the remaining erythrocytes. The cells were washed with PBS, adjusted to 2×10^7 cells/ml and kept on ice until further use.

Effect of calixarenes on cellular mortality

Human neutrophils were purified from citrated venous blood freshly isolated from healthy volunteers using a 33% (v/v) Hypaque–Ficoll gradient. After 20 min centrifugation at 800 g at 20 °C, the pellet was submitted to a hypotonic lysis for 5–15 min on ice [26]. After 5 min centrifugation at 350 g at 4 °C, the neutrophil pellet was collected and washed once in PBS. Cells (1×10^6 cells in 250 µl PBS) were incubated in presence of calixarene (100 nM or 10 µM) or PBS for 30 min at 37 °C.

Then, cell viability was evaluated by Trypan blue (0.07%) exclusion. Apoptotic and necrotic cells were discriminated by using Annexin-V-FITC and PI labelling. Briefly, cells (1×10^6 PMN) were washed with PBS and centrifuged at 200×*g* for 5 min. Cells were re-suspended in 100 µl incubation buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$) containing either Annexin-V-FITC solution or PI (1 µg/ml). After 15 min incubation at room temperature, 0.4 ml incubation buffer was added and cells were analysed by flow cytometry. Fluorescence intensity (FL1 of the Annexin-V-FITC labelled PMNs and FL2 of PI labelled PMN) was measured on a FACScalibur (Becton Dickinson) cytometer.

Measurement of NADPH oxidase activity

Superoxide production was measured by chemiluminescence [27]. The 1×10^6 neutrophils (50 µl) purified from Buffy-coat were suspended 200 µl PBS containing 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 20 mM glucose, 20 µM luminol, and 10 U/ml of horseradish peroxidase. Superoxide production was measured after addition of 10 µl PMA (130 nM final concentration) (positive control), 10 µl calixarenes (100 nM or 10 µM final concentration) or PBS. Photon emission was recorded at 37 °C during 30 min with a Luminoskan (Labsystem, Pontoise, France). In some experiments, at the end of the measurement, 10 µl PMA (130 nM final concentration) was added and photon emission was recorded again for 30 min. Results were expressed as the sum of relative luminescence unit (RLU).

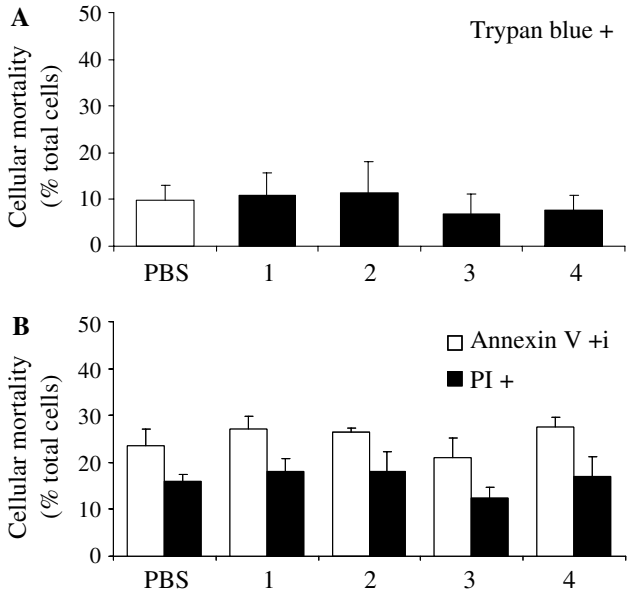

Figure 1. Effect of pre-treatment with 10 µM calixarenes on neutrophil viability. Neutrophils (1×10^6 cells in 250 µl) were incubated with PBS or 10 µM calix[4]arene (1), *para*-Sulphonato-calix[4]arene (2), *para*-Sulphonato-calix[6]arene (3), *para*-Sulphonato-calix[8]arene (4) for 30 min at 37 °C. (A) After incubation, PMN were incubated with trypan blue (0.07%) and counted. Blue-stained cells corresponding to trypan blue-permeable cells were counted and expressed as a percentage of total cells. (B) Flow cytometry of calixarene-pre-treated PMN labelled with Annexin-V-FITC or PI as described in the Materials and methods. The fluorescence of 10,000 cells was measured. Necrotic cells are positively stained with both PI and Annexin-V while apoptotic cells are only positively stained with Annexin-V-FITC. The data are representative of two independent experiments.

Results and discussion

In order to determine the effect of certain calix[*n*]arenes on PMNs, different parameters were studied. On one hand, cell viability was studied by Trypan blue exclusion and by flow cytometry. On the other hand, the potential toxic effect of these molecules was determined by evaluating the neutrophil stimulation in terms of NADPH oxidase activation.

Results of viability experiments, reported in Figure 1A, show no significant difference between cells incubated in PBS, used as control, and cells incubated in calixarene solution. Trypan blue exclusion allows to discrimination between intact and permeabilized cells, but not cells in the early stages of apoptosis. To be sure that calixarenes do not induce cell apoptosis, we performed an additional assay to detect apoptotic and necrotic cells, using flow cytometry. The presence of phosphatidylserine on the outer leaflet of apoptotic cells was analysed using fluorescent annexin V. Since phosphatidylserine can also be exposed on the outer leaflet of necrotic cells, we used simultaneously PI, a DNA stain of permeabilized cells. Cells positive for Annexin V (Annexin V+) labelling represent both apoptotic and necrotic cells (Figures 1B and 2). No significant difference in cell viability (75–80% of total cells) was observed between control cells incubated in PBS and cells incubated with the calix[*n*]arene derivatives (Figures 1B and 2). Then, necrosis and apoptosis were analysed separately. Cells only positive for Annexin V (Annexin V+ and PI−) labelling correspond to apoptotic cells while cells only positive for propidium iodide (PI+ and Annexin V−) labelling are necrotic cells. Results show that both apoptosis (10–15% of total cells) and necrosis (10–15% of total cells) were unchanged upon cell-incubation with calixarenes as compared to control cells (Figures 1B, 2 and 3).

Studies of the reaction of PMNs towards the calix[*n*]arene derivatives were based on the measurement of superoxide production by these cells in response to

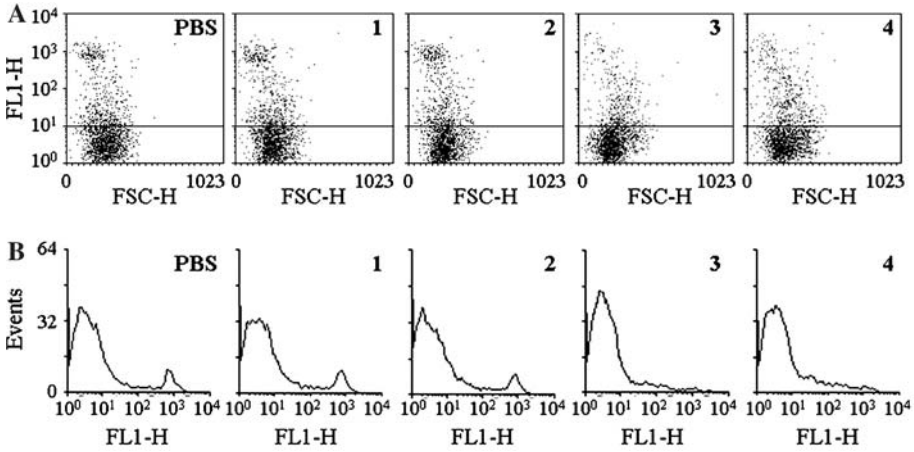

Figure. 2. Flow cytometry analysis of cellular viability. Neutrophils (1×10^6 cells in 250 µl) were incubated with PBS or 10 µM calix[4]arene (1), *para*-Sulphonato-calix[4]arene (2), *para*-Sulphonato-calix[6]arene (3), *para*-Sulphonato-calix[8]arene (4) for 30 min at 37 °C. PMN were stained with Annexin-V-FITC. (A) Fluorescence (FL1-H) of 10,000 cells was measured by flow cytometry. (B) Cells with a fluorescence superior to 10^1 were considered positively stained. The data are representative of two independent experiments.

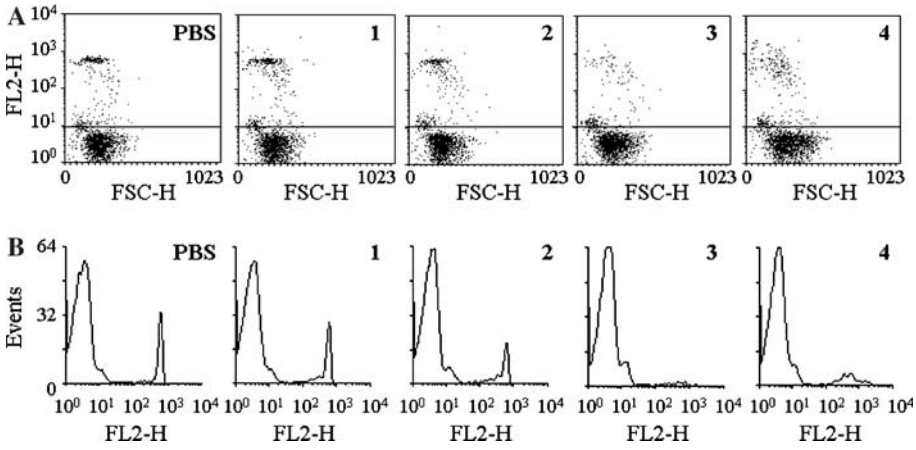

Figure 3. Flow cytometry analysis of cellular necrosis. Neutrophils (1×10^6 cells in 250 µl) were incubated with PBS or 10 µM calix[4]arene (1), *para*-Sulphonato-calix[4]arene (2), *para*-Sulphonato-calix[6]arene (3), *para*-Sulphonato-calix[8]arene (4) for 30 min at 37 °C. PMNs were stained with PI to determine necrotic cells. (A) Fluorescence (FL2-H) of 10,000 cells was measured by flow cytometry. (B) Cells with a fluorescence superior to 10^1 were considered positively stained. The data are representative of two independent experiments.

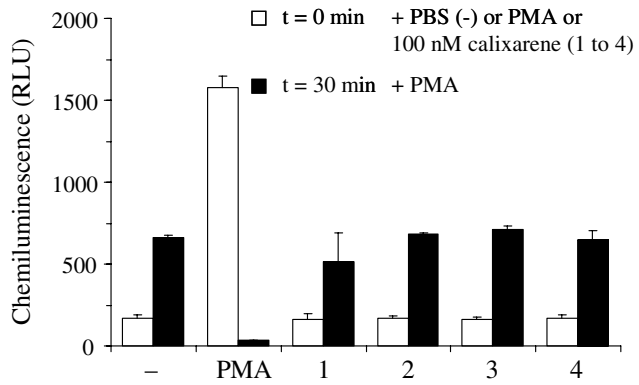

Figure 4. Effect of pre-treatment with 100 nM calixarenes on NADPH oxidase activity in PMA-stimulated neutrophils. PMNs (1×10^6 neutrophils) suspended in PBS containing luminol, glucose, horseradish peroxidase (as described in the Materials and methods) were incubated with 100 nM calix[4]arene (1), *para*-Sulphonato-calix[4]arene (2), *para*-Sulphonato-calix[6]arene (3), *para*-Sulphonato-calix[8]arene (4) or PBS (–) or PMA at 130 nM (PMA) (open bars). Photon emission was recorded at 37 °C during 30 min. At the end of the incubation, 10 µl PMA (130 nM final concentration) was added to each well and photon emission was measured for another 30 min-duration at 37 °C (black bars). Results are expressed as the average of two experiments (± SD).

stimulation. This experiment was carried out in two steps: first, cells were incubated 30 min with PBS as negative control, PMA at 130 nM as positive control and different calixarene derivatives at 100 nM as assays. Superoxide production was measured during the 30 min incubation by chemiluminescence (Figure 4 – open bars). Then, PMA (130 nM) was added in each well and superoxide release within another 30 min incubation was determined. Results are reported in Figure 4. In the first 30 min, neutrophils are not stimulated by *para*-H-calix[4]arene nor by *para*-Sulphonato-calix[*n*]arenes: the cellular response was the same as obtained with PBS, used as negative control. After PMA addition, these cells keep their NADPH oxidase activation capabilities: superoxide production was not affected by the pre-treatment with calixarene derivatives. Then, PMNs treated with 100 nM calixarenes are still able to produce O_2^- in response to PMA stimulation while PMNs firstly stimulated by PMA are no more able to respond to a second PMA stimulation. Data clearly indicate that neutrophil membrane was not affected (permeabilized) by the calixarene treatment.

We also analysed the neutrophil kinetic response after calixarene and PMA stimulation (Figure 5). The results show that pre-treatment with the calix[*n*]arene derivatives does not affect the response of the PMNs during the incubation. Indeed, no effect of treatment at a 100 nM concentration of the various calix[*n*]arenes was observed on the kinetic of PMA-dependent NADPH oxidase activation in PMNs.

Similar results were obtained with a higher calixarene concentration (10 µM) suggesting an absence of cytotoxicity of these derivatives on neutrophils in our experimental conditions (Figure 6).

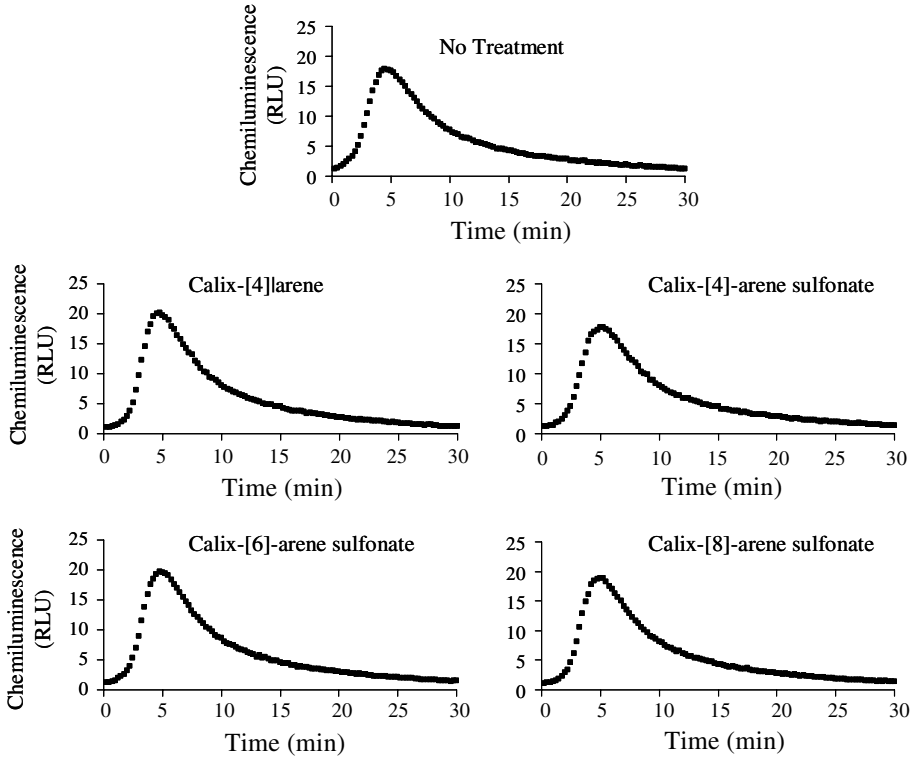

Figure 5. Kinetic of NADPH oxidase activation in PMNs pre-treated with 100 nM calixarenes and stimulated with PMA. PMNs (1×10^6 neutrophils) suspended in PBS containing luminol, glucose, horseradish peroxidase (as described in the Materials and methods) were incubated with 100 nM calix[4]arene, *para*-Sulphonato-calix[4]arene, *para*-Sulphonato-calix[6]arene, *para*-Sulphonato-calix[8]arene or PBS, 30 min at 37 °C. At the end of the incubation, 10 µl PMA (130 nM final concentration) was added to each well. Photon emission was recorded at 37 °C during 30 min.

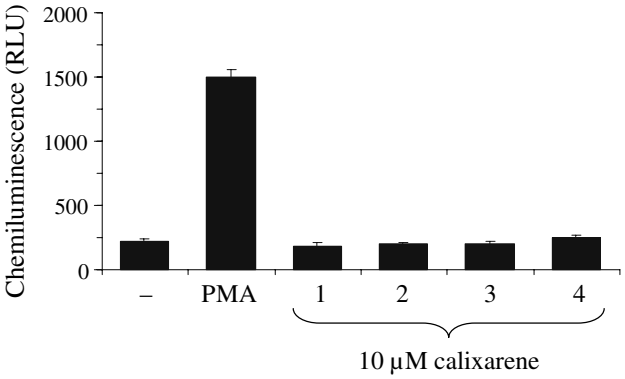

Figure 6. Effect of 10 µM calixarenes treatment on NADPH oxidase activity in PMNs. PMNs (1×10^6 neutrophils) suspended in PBS containing luminol, glucose, horseradish peroxidase (as described in the Materials and methods) were incubated with 10 µM calix[4]arene (1), *para*-Sulphonato-calix[4]arene (2), *para*-Sulphonato-calix[6]arene (3), *para*-Sulphonato-calix[8]arene (4), PBS (–) or PMA, 30 min at 37 °C. Photon emission was recorded during incubation at 37 °C. Results are expressed as the average of two experiments (±SD).

Conclusion

In the present study, we evaluated the effect of novel calixarene derivatives on the viability and stimulation of neutrophils. These cells, involved in host defence, are rapidly mobilized and stimulated when host elements are detected in an organism. Activation of the phagocyte NADPH oxidase, present in the granule and plasma membrane of these cells, is a good tool to evaluate the degree of neutrophil stimulation. In our experiments, cell viability was not modified in presence of certain calix[*n*]arene derivatives. Moreover, the calixarenes do not induce NADPH oxidase activation in neutrophils and do not alter the capacity of neutrophil to respond to PMA. All these results suggest that the *para*-H-calix[4]arene and the *para*-Sulphonato-calix[*n*]arenes (with $n=4,6$ or 8) are not cytotoxic. Even if they need further *in vitro* evaluation, these calixarene derivatives prove to be good candidates for bio-pharmaceutical applications.

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