Inflammation, Vol. 39, No. 1, February 2016 (© 2015)
DOI: 10.1007/s10753-015-0264-4

ORIGINAL ARTICLE

Activation of Neutrophils via IP_3 Pathway Following Exposure to *Demodex*-Associated Bacterial Proteins

Fred McMahon,[1] Nessa Banville,[2] David A. Bergin,[2] Christian Smedman,[3] Staffan Paulie,[3] Emer Reeves,[2] and Kevin Kavanagh[1,4]

Abstract—Rosacea is a chronic inflammatory condition that predominantly affects the skin of the face. Sera from rosacea patients display elevated reactivity to proteins from a bacterium (*Bacillus oleronius*) originally isolated from a *Demodex* mite from a rosacea patient suggesting a possible role for bacteria in the induction and persistence of this condition. This work investigated the ability of *B. oleronius* proteins to activate neutrophils and demonstrated activation via the IP_3 pathway. Activated neutrophils displayed increased levels of IP_1 production, F-actin formation, chemotaxis, and production of the pro-inflammatory cytokines IL-1β and IL-6 following stimulation by pure and crude *B. oleronius* protein preparations (2 μg/ml), respectively. In addition, neutrophils exposed to pure and crude *B. oleronius* proteins (2 μg/ml) demonstrated increased release of internally stored calcium (Ca^{2+}), a hallmark of the IP_3 pathway of neutrophil activation. Neutrophils play a significant role in the inflammation associated with rosacea, and this work demonstrates how *B. oleronius* proteins can induce neutrophil recruitment and activation.

KEY WORDS: *Bacillus*; *Demodex*; inflammation; neutrophils; rosacea.

INTRODUCTION

Rosacea is a chronic inflammatory dermatosis that affects the skin of the face, the eyelid margins, and the corneal surface [1–3]. A number of forms of rosacea have been recognized and include papulopustular, ocular, phymatous, and erythematotelangiectatic [4, 5]. While the etiology of rosacea is complex and may involve a variety of factors, treatment of the condition has relied upon the use of antibiotics (e.g., metronidazole and tetracycline) [6–8], and therapy is characterized by periods of remission and relapse [3]. The efficacy of antibiotics in controlling the condition may be due to their anti-inflammatory properties, although anti-inflammatory agents such as steroids and tacrolimus are not effective in treating the condition and their use can lead to deterioration of the condition [6].

Neutrophils play a central role in the innate immune response against pathogens, and a role in the inflammation which is characteristics of rosacea has been suggested [9]. Many agents used in the treatment of rosacea, such as tetracyclines, azelaic acid, retinoids, and metronidazole [9–11], inhibit reactive oxygen species (ROS) production by neutrophils and lead to clearance of the condition. Neutrophils may also induce tissue damage by the release of proteolytic enzymes during degranulation which can degrade collagen [12, 13] and activate key signaling pathways [14, 15] which can result in inflammation.

Electronic supplementary material The online version of this article (doi:10.1007/s10753-015-0264-4) contains supplementary material, which is available to authorized users.

[1] Department of Biology, Maynooth University, Co. Kildare, Ireland
[2] Respiratory Research Division, Department of Medicine, Royal College of Surgeons in Ireland, Beaumont Hospital, Dublin 9, Ireland
[3] Mabtech AB, Nacka Strand, Sweden
[4] To whom correspondence should be addressed at Department of Biology, Maynooth University, Co. Kildare, Ireland. E-mail: kevin.kavanagh@nuim.ie

The regulation of intracellular neutrophil signal transduction for chemotaxis and degranulation requires an increase in the cytosolic Ca^{2+} [16]. Chemoattractant signaling molecules such as fMLP or IL-8 bind to a G-protein-coupled receptor (GPCR) leading to the activation of the β isoform of phospholipase C (PLC), and the generation of the second messenger, inositol 1,4,5-triphosphate (IP_3). This results in the rapid release of stored Ca^{2+} from Ca^{2+}-gated ion channels of calciosomes, and neutrophil activation [17]. The signaling cascade for the release of internally stored Ca^{2+} following the generation of IP_3 has been proposed as a potential therapeutic target against the inflammatory response of neutrophils in a range of diseases [16, 18, 19].

Rosacea patients demonstrate an elevated density of *Demodex* mites in their skin compared to controls [20–22]. The nature of the sebum produced on the faces of rosacea patients is different to that in controls [23], and this may facilitate the growth in the *Demodex* population. It has been suggested that the elevated density of *Demodex* mites may irritate the lining of the pilosebaceous unit and initiate an inflammatory response [3].

A number of studies have suggested that bacteria may play a role in the induction of rosacea [24–26]. A bacterium (*Bacillus oleronius*) was isolated from a microdissected *Demodex* mite extracted from the face of a papulopustular rosacea patient [25], and this bacterium was previously isolated from the digestive tract of a termite where it may facilitate digestion [27]. This bacterium has also been isolated from *Demodex* mites and eyelashes of patients with blepharitis [28] and is sensitive to the antibiotics used to treat rosacea [25]. Sera from patients suffering from papulopustular [25], ocular [29], and erythematotelangiectatic [30] rosacea demonstrated elevated reactivity to proteins isolated from *B. oleronius*, thus suggesting a possible link between the presence of this bacterium and rosacea [2, 3, 25]. A positive correlation between serum reactivity to *Bacillus* protein, elevated *Demodex* density, and altered sebum production has been established [31]. It has previously been demonstrated that exposure of a corneal epithelial cell line to *Bacillus* proteins induced cell migration and an aberrant wound healing response [32]. The "crude" *B. oleronius* protein preparation [32, 33] used here consists of all the *B. oleronius* proteins that might be released from dead *Demodex* while the "pure" protein preparation consists of the *B. oleronius* proteins to which rosacea patients show serum reactivity [25, 29, 30].

Exposure of neutrophils to proteins from *B. oleronius* resulted in degranulation and the production of inflammatory cytokines [34]. The aim of the work presented in this study was to characterize the response of neutrophils to *Bacillus* proteins as this may explain how the release of these proteins from *Demodex* mites in the pilosebaceous unit could lead to neutrophil activation and inflammation.

MATERIALS AND METHODS

Preparation of Bacterial Protein Preparations

B. oleronius cells were incubated at 30 °C and 200 rpm in nutrient broth (Oxoid) under aerobic conditions to the stationary phase, and proteins were extracted and purified as described [25]. The protein preparation was purified by anion exchange separation by ÄKTA-FPLC as described [33]. Fractions corresponding to the proteins of interest were pooled, precipitated (termed pure *B. oleronius* protein) and resuspended in phosphate-buffered saline (PBS) at a concentration of 200 μg/ml. Based on previous studies investigating the effects of the *B. oleronius* protein preparations, two doses (2 and 6 μg/ml) of the crude and pure protein preparations were employed here [25, 33, 34].

Isolation of Neutrophils

Human neutrophils were isolated from healthy volunteers by dextran (10 %, *w/v*; Sigma) sedimentation and Ficoll-Hypaque centrifugation (Axis-Shield PoC AS, Oslo, Norway) as described [35]. Cell viability was assessed by the trypan blue exclusion assay [36]. Ethical approval for the isolation of human neutrophils was granted by NUIM ethics committee.

Quantification of D-myo-Inositol-1-Phosphate

The quantity of IP_1 in neutrophils was assessed using an IP-One ELISA assay kit (Cisbio Bioassays, Codolet, France). Freshly isolated human neutrophils (5×10^6/ml) were re-suspended in stimulation buffer, and stimulants were added (N-formyl-methionyl-leucyl-phenaylalnine (fMLP; positive control), 1 μM; interleukin (IL)-8 (positive control), 10 ng/ml (positive control); crude *B. oleronius* protein, 2 and 6 μg/ml; and pure *B. oleronius* protein, 2 and 6 μg/ml) at 37 °C in a 5 % CO_2 humidified atmosphere for 1 h. After development, the ELISA plate was read at absorbance 450 nm on a BioTek Synergy™ HT (BioTek Instruments Inc., Vermont,

USA) microplate reader to generate a standard curve and quantify levels of IP_1.

Quantification of Intracellular Ca^{2+} Flux in Neutrophils

Intracellular calcium (Ca^{2+}) levels of neutrophils exposed to IL-8 (10 ng/ml), fMLP (1 μM), crude *B. oleronius* protein (2 μg/ml), pure *B. oleronius* protein (2 μg/ml), and unstimulated neutrophils were measured using the Fluo-4 NW calcium assay kit (Molecular Probes®, Invitrogen™ Detection Technologies). Ca^{2+} fluorescence was determined with excitation/emission at 494/516 nm, and read at 10-s intervals for a total of 90 s with stimulation of human neutrophils occurring after 40 s, indicated by a downward arrow, using a microplate reader.

F-Actin Polymerization Following Exposure of Neutrophils to *Bacillus* Proteins

F-actin and G-actin abundance and distribution were assessed according to the method described by [37]. Neutrophils were isolated and resuspended at a density of 1×10^7 cells per treatment and stimulated with IL-8 (10 ng/ml), crude *B. oleronius* protein (2 and 6 μg/ml), or pure *B. oleronius* protein (2 and 6 μg/ml), at 37 °C for 10 min.

Equal volumes of extracted sub-cellular neutrophil fractions were loaded onto a 12.5 % SDS-PAGE gel and separated under reducing conditions, resolved by electrophoresis, transferred onto a nitrocellulose membrane (Thermo Scientific™, Fisher Scientific Ltd., Ireland), and probed for G- and F-actin abundance (Anti-Actin, Clone C4, Millipore, Germany), followed by an anti-human HRP-linked secondary antibody (HRP-linked mouse antiserum). Immunoreactive protein bands were visualized by incubating the membranes for 10 min in diaminobenzidine tetrahydrochloride [DAB; 1 mg/l in 100 mM Tris–HCl (pH 7.6) containing 15 μl of hydrogen peroxide] before washing in distilled water and drying. Immunobands were quantified by densitometry using ImageJ software (NIH, MD, USA).

Measurement of Neutrophil Chemotaxis

Chemotaxis assays were performed using a multiwell chemotaxis chamber (Neuro Probe, Inc., MD, USA), and a 5 μM pore filter (Neuro Probe) with the stimulants (fMLP, 1 μM; IL-8, 10 ng/ml; crude *B. oleronius* protein, 2 μg/ml; pure *B. oleronius* protein, 2 μg/ml) added to the bottom of the chamber. Cells that had completely migrated through the filter were counted in five random high-power fields of vision (Magnification 40×) per well per treatment by light microscopy.

Pro-inflammatory Cytokine Enzyme-Linked Immunosorbent Assay (ELISA)

Isolated human neutrophils (1×10^6/ml in RPMI-1640 GlutaMAX™ media (Gibco®) supplemented with 5 % (*v*/*v*) FCS) were added to each well of a 24-well flat-bottomed tissue culture plate (Falcon™, BD Biosciences, UK) and incubated for 16 and 24 h. Cells were treated with 2 and 6 μg/ml of the crude *B. oleronius* protein and pure *B. oleronius* protein, and IL-8 (10 ng/ml). Cell culture supernatants were harvested and the levels of IL-1β and IL-6 were measured using commercial ELISA kits (Mabtech Ab) according to the manufacturer's guidelines. The plates were read at 405 nm for the IL-1β ELISA, and at 450 nm for the IL-6 ELISA on a microplate reader.

Statistical Methods

The statistical significance was assessed using Prism5 software (GraphPad Software, San Diego, CA, USA). Data are presented as the mean±standard error. Statistical analysis was performed by paired Student's *t* test. A *p* value less than 0.05 was deemed statistically significant.

RESULTS

Exposure of Neutrophils to *Bacillus* Proteins Leads to Increased D-*myo*-Inositol 1-Phosphate (IP_1) Levels

The quantification of IP_1 in neutrophils was performed as described as this can be used as a measure of IP_3, a second messenger involved in neutrophil signaling [17]. An increase in IP_1 production was observed following exposure of neutrophils to the pure *B. oleronius* protein preparation at 2 μg/ml (4.7-fold, $p=0.016$) and 6 μg/ml (4.4-fold, $p=0.02$; Fig. 1). Exposure of neutrophils to the crude *B. oleronius* protein extract led to an increase of IP_1 in 2 μg/ml (13.9-fold; $p=0.011$) and 6 μg/ml (10.9-fold; $p=0.032$) treatments, and these values were similar to IP_1 levels detected for neutrophils stimulated with fMLP (1 μM; 13.8-fold; $p=0.0146$) and IL-8 (10 ng/ml; 8.5-fold; $p=0.0046$; Fig. 1).

Calcium Efflux in Neutrophils Exposed to *Bacillus* Protein

The activation of neutrophils is characterized by the rapid movement of intracellular Ca^{2+} ions stored in the

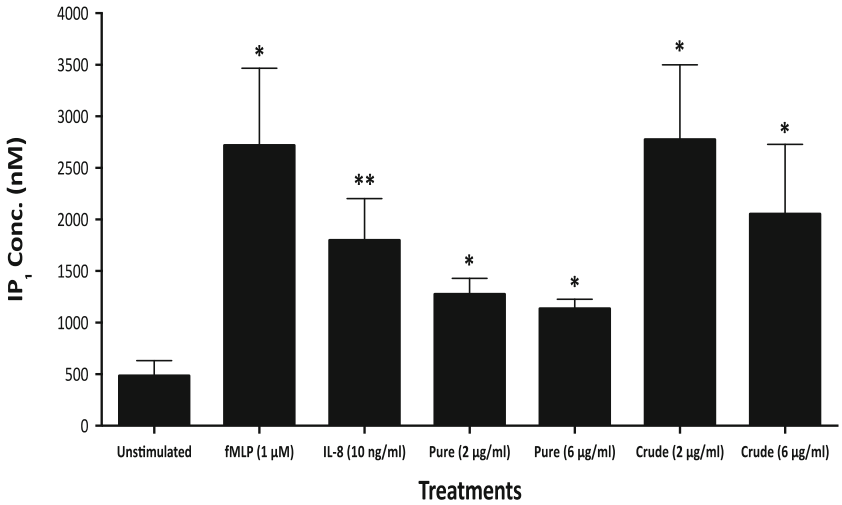

Fig. 1. Effect of *B.oleronius* protein preparation on IP_1 formation in neutrophils. Neutrophils were exposed to PBS (unstimulated), fMLP (1 μM), IL-8 (10 ng/ml), pure *B. oleronius* protein (2 and 6 μg/ml), and crude *B. oleronius* protein (2 and 6 μg/ml) for 10 min, and the effect on IP_1 formation in cytosols was quantified. Significance: $*p<0.05$, $**p<0.01$.

calciosomes induced by IP_3 receptor binding [38–41]. Stimulation of neutrophils with *B. oleronius* protein preparations resulted in a rise in released Ca^{2+} (Fig. 2). The increase in Ca^{2+} levels was significant following exposure of neutrophils to the crude *B. oleronius* protein preparation (2 μg/ml; 1.4-fold; $p=0.030$) at 10 s. At 40 s post-stimulation, Ca^{2+} flux was increased in neutrophils exposed to crude *B. oleronius* protein (1.5-fold; $p=0.021$), pure *B. oleronius* protein (1.3-fold; $p=0.003$), and IL-8 (1.3-fold; $p=0.003$). Stimulation of neutrophils with fMLP (1 μM) resulted in an increase in Ca^{2+} levels at 10 (1.1-fold), 20 (1.2-fold), 30 (1.2-fold), and 40 (1.2-fold) seconds post-stimulation but was not statistically significant.

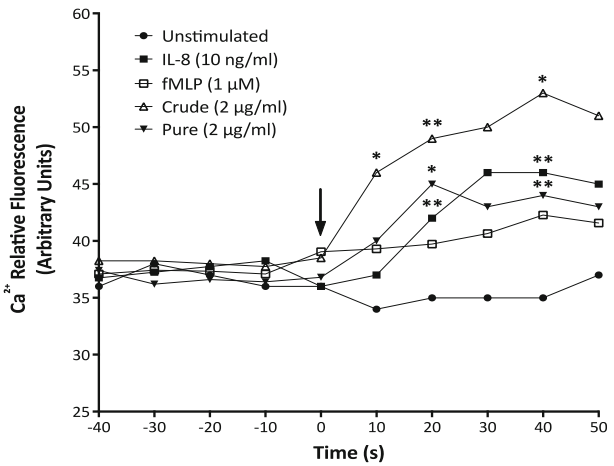

Fig. 2. Effect of *B. oleronius* protein preparation on calcium flux in neutrophils. Isolated neutrophils were exposed to PBS (unstimulated), IL-8 (10 ng/ml), fMLP (1 μM), crude *B. oleronius* protein extract (2 μg/ml), or pure *B. oleronius* protein extract (2 μg/ml), and the effect on cytosolic Ca^{2+} flux was measured. Stimulation of neutrophils by the different treatments occurred at 0 s (indicated by *arrow*). Significance: $*p<0.05$, $**p<0.01$. (Figure is a representative of one repeat of the assay performed in triplicate, and the statistics are representative of the assay performed in triplicate relative to the unstimulated control.)

Cytoskeletal Reassembly in Neutrophils Induced by *Bacillus* Proteins

The ability of activated neutrophils to perform the morphological and functional changes required to phagocytose and kill an opsonized pathogen is dependent upon the conversion of G- to F-actin [42]. Neutrophils were exposed to *Bacillus* protein extracts for 10 min, and the relative abundance of G-actin and F-actin was quantified by Western blot (Fig. 3a), and the distribution ratio was calculated (Fig. 3b, C). In unstimulated neutrophils, the abundance of G-actin exceeded that of F-actin by 2.6-fold. Stimulation of cells with IL-8 (10 ng/ml) led to an increase in F-actin (1.7-fold; $p=0.025$) and a relative decline in G-actin (0.7-fold; $p=0.028$), demonstrating the cytoskeletal conversion of G-actin to F-actin. Neutrophils exposed to pure *B. oleronius* protein preparation (2 and 6 μg/ml) showed significantly elevated levels of F-actin (2.0-fold, $p=0.011$, and 1.5-fold, $p=0.024$, respectively) and reduced abundance of G-actin (0.6-fold, $p=0.048$, and 0.8-fold, $p=0.025$, respectively). The increase in F-actin following exposure of neutrophils to pure *B. oleronius* protein at 6 μg/ml was calculated to be significant compared to pure *B. oleronius* protein at 2 μg/ml ($p=0.019$),

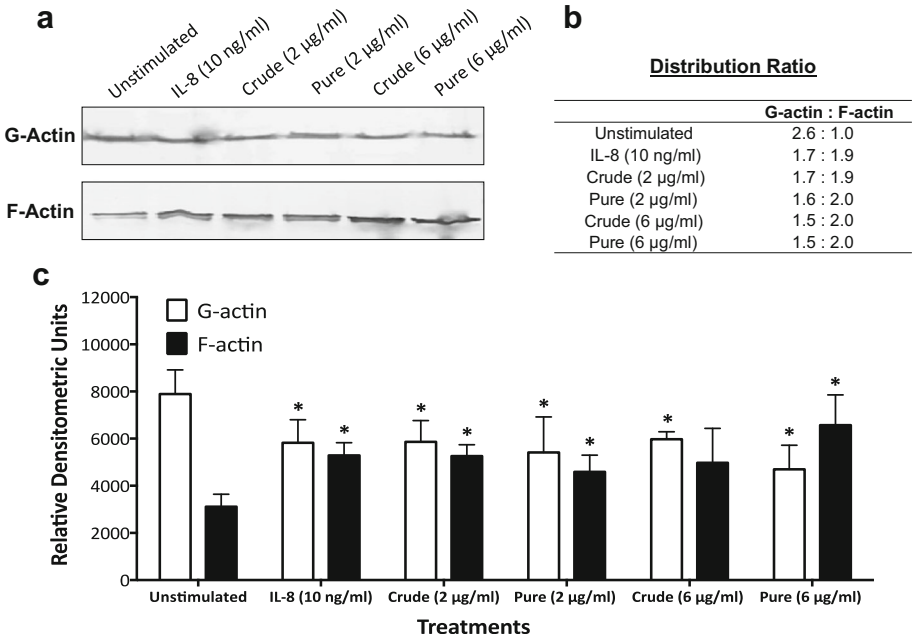

Fig. 3. Alteration in G/F-actin ratio in neutrophils exposed to *B. oleronius* protein preparations. The abundances of G-actin and F-actin were assessed for each treatment by Western blot (**a**), the distribution ratio of G-actin/F-actin was calculated (**b**), and the abundance of G-actin and F-actin for each treatment was quantified using ImageJ densitometric software (**c**). Significance: *$p<0.05$.

and crude *B. oleronius* protein at 6 µg/ml ($p=0.008$). The relative levels of F-actin were increased in neutrophils exposed to the crude *B. oleronius* protein preparation with a significant increase of F-actin, 1.4-fold, at 2 µg/ml ($p=0.04$). Exposure of neutrophils to crude *B. oleronius* protein at 6 µg/ml induced a 1.7-fold increase in F-actin but did not reach significance ($p=0.0557$). The abundance G-actin declined significantly following exposure of neutrophils to crude *B. oleronius* protein preparation at 2 and 6 µg/ml (0.7-fold, $p=0.0165$, and 0.7-fold, $p=0.0147$, respectively; Fig. 3c).

Induction of Neutrophil Chemotaxis in Response to *Bacillus* Proteins

The effect of *B. oleronius* crude (2 µg/ml) and pure protein (2 µg/ml) preparations on neutrophil chemotaxis was investigated. The results demonstrated that exposure to IL-8 (10 ng/ml) led to a 2.9-fold ($p<0.0001$) increase in neutrophil chemotaxis. Exposure to the crude *B. oleronius* protein preparation or to the pure *B. oleronius* protein preparation induced a 3.5-fold ($p<0.0001$) or 3.9-fold ($p<0.0001$) increase in migration of neutrophils toward a chemotactic gradient, respectively (Fig. 4).

Exposure of Neutrophils to *Bacillus* Proteins Leads to Elevated IL-1β and IL-6 Secretion

The effect of *B. oleronius* protein preparations on the ability of neutrophils to secrete IL-1β and IL-6 was investigated. IL-1β is a pro-inflammatory cytokine and also functions in cell proliferation and the induction of apoptosis [43]. IL-6 is a pro-inflammatory cytokine associated with the response to bacterial infection, and high levels of expression have been recorded in psoriatic skin [44].

Neutrophils exposed to pure *B. oleronius* protein (2 and 6 µg/ml) demonstrated significant increases in IL-1β production after 16 (201 and 150 pg/ml, respectively) and 24 (89 and 83 pg/ml, respectively) hours of incubation ($p<0.0001$; Fig. 5a). There was a significant increase in IL-1β secretion by neutrophils exposed to pure *B. oleronius* protein at 2 µg/ml ($p=0.0005$), and at 6 µg/ml ($p=0.0003$) after 16 h, and after 24-h exposure at 2 µg/ml ($p=0.0009$) and at 6 µg/ml ($p<0.0001$) pure *B. oleronius* protein. In contrast, cells exposed to the crude *B. oleronius* protein preparation (2 and 6 µg/ml) showed no alteration in IL-1β secretion (Fig. 5a).

Neutrophils exposed to 2 or 6 µg/ml pure or crude *B. oleronius* protein preparation demonstrated increased secretion of IL-6 production ($p<0.001$) with the highest amount occurring at 24 h (Fig. 5b). IL-6 production

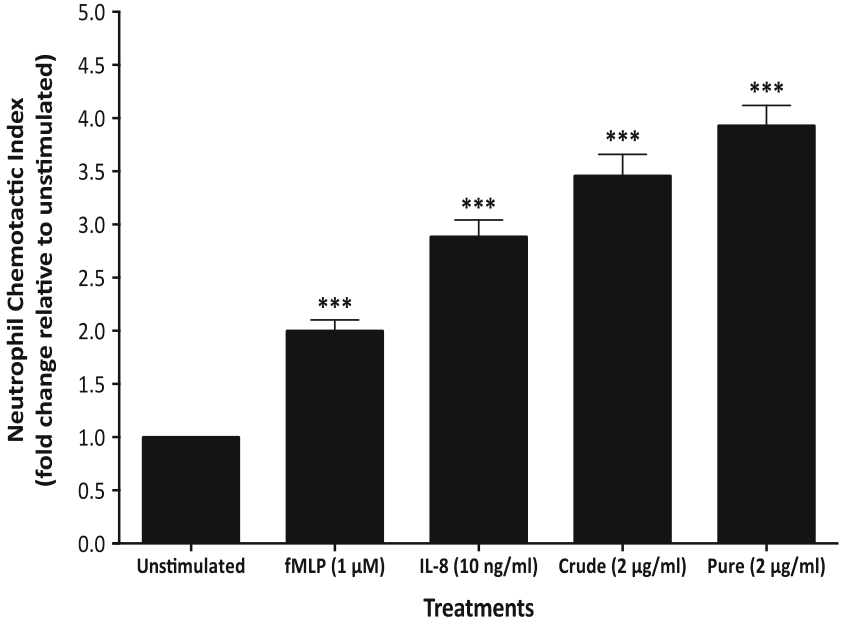

Fig. 4. Effect of *B. oleronius* protein preparations on neutrophil chemotaxis. Neutrophils were isolated and exposed fMLP (1 μM), IL-8 (10 ng/ml), pure *B. oleronius* protein (2 μg/ml), and crude *B. oleronius* protein (2 μg/ml) for 30 min, and the effect on neutrophil migration along a chemotactic gradient was quantified. Significance: ***$p<0.001$.

increased 2.5-fold and 2.2-fold following exposure of neutrophils to 2 and 6 μg/ml pure *B. oleronius* protein ($p<0.0001$, and $p=0.0048$, respectively) after 16 h. Treatment of neutrophils with the crude *B. oleronius* protein preparation at 2 and 6 μg/ml produced similar levels of IL-6 compared to IL-6 production following stimulation by IL-8. After 24 h, IL-6 production increased 1.8-fold and 1.9-fold following exposure of neutrophils to pure

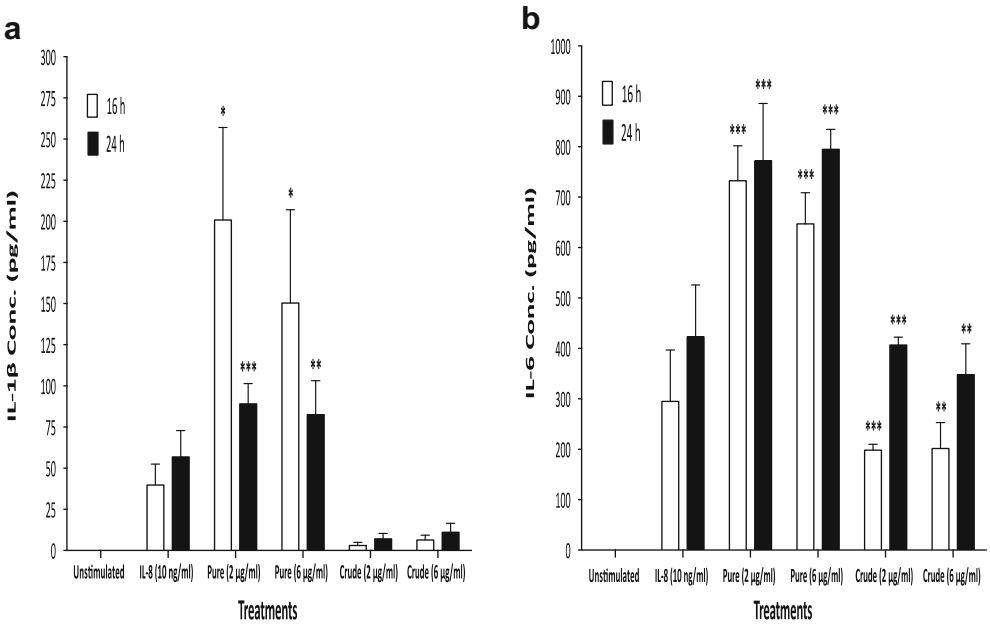

Fig. 5. Production of IL-1β and IL-6 by neutrophils exposed to *B. oleronius* protein preparations. Neutrophils were exposed to IL-8 (10 ng/ml), pure *B. oleronius* protein (2 and 6 μg/ml), or crude *B. oleronius* protein preparation (2 and 6 μg/ml), for 16 and 24 h. The secretion of the pro-inflammatory cytokines, IL-1β (**a**) and IL-6 (**b**), was measured by ELISA. Significance: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

B. oleronius protein ($p=0.0001$, and $p=0.0018$, respectively). Exposure of neutrophils to crude *B. oleronius* protein at 2 and 6 μg/ml showed similar levels of IL-6 compared to the production of IL-6 following stimulation by IL-8 (positive control).

DISCUSSION

The role of *Demodex* mites in the biology of the skin has been intensely studied by dermatologists and scientists since their discovery by Henle and Berger (1841) [45]. Akilov and Mumcuoglu [46] showed that individuals with the HLA Cw2 and Cw4 haplotypes were more susceptible to increased *Demodex* mite infestation in the skin and have a decreased natural killer T cell population. Casas *et al.* [47] quantified *D. folliculorum* mite colonization in erythematotelangiectatic rosacea and papulopustular rosacea patients and investigated the activation of a skin innate immune response. They demonstrated that genes coding for IL-8, IL-1β, TNF-α, and Cox-1 were increased, and observed elevated expression of genes coding for the inflammasome.

Features of the skin microenvironment including temperature, pH, and sebum composition could influence *Demodex* mite population development and thus the onset of symptoms associated with rosacea [2, 23, 48–50]. Murillo and colleagues [51] characterized the microbiota of *Demodex* mites isolated from rosacea patients and identified a wide range of bacteria in the mites. Studies have demonstrated high levels of rosacea patient sera reactivity to proteins produced by *B. oleronius*, a bacterium isolated from a *Demodex* mite from a patient with papulopustular rosacea, indicating a possible role for this or closely related bacteria in the induction of rosacea [25, 29–31]. Neutrophil influx and activation are considered a key factor in the induction of inflammation in the skin of rosacea patients [6, 8]. We have previously demonstrated that neutrophils exposed to *B. oleronius* proteins showed increased migration and elevated release of MMP-9 and cathelicidin [34]. This may contribute to the tissue damage and inflammation evident in areas of skin characteristic of rosacea. In the current work, the process which results in neutrophil activation has been characterized.

Analysis of the *B. oleronius* protein preparations used here confirmed the absence of LPS contamination (see supplementary file, Figs. S1, S2, and S3), thus indicating that effects observed here were not attributable to endotoxin presence. Neutrophils exposed to the *B. oleronius* protein preparations demonstrated elevated levels of IP_1 indicating the activation of GPCR signaling leading to the generation of the secondary messenger IP_3 [17] (Fig. 1). Neutrophils activated through the IP_3 pathway exhibit a rapid release of internally stored Ca^{2+} following the occupancy of IP_3 and its receptors on the Ca^{2+}-gated ion channels of the calciosomes, with a return to the basal level of cystolic Ca^{2+} by the action plasma membrane and sacro(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pumps [16, 17, 52–54]. The results presented here indicate that exposure of neutrophils to *B. oleronius* protein preparations leads to a rapid release of stored Ca^{2+} which begins to decline 50 s after the application of the proteins (Fig. 2). Following stimulation of neutrophils by the *B. oleronius* proteins, activated neutrophils also demonstrated elevated levels of F-actin (Fig. 3), increased chemotaxis, indicating movement toward the *Bacillus* proteins (Fig. 4), and increased production of the pro-inflammatory cytokines, IL-1β and IL-6 (Fig. 5a, b).

One scenario that might explain the follicular-orientated inflammation characteristic of papulopustular rosacea suggests the release of antigenic bacterial proteins from dead *Demodex* mites within the pilosebaceous unit. These are released from the pilosebaceous unit, attracting neutrophils to the surrounding area. Activated neutrophils degranulate, releasing MMP-9 and cathelicidin which cause tissue damage. Neutrophils also produce elevated levels of IL-8 and TNF-α attracting more neutrophils to the vicinity and induce an exaggerated inflammatory response [34]. The current work demonstrates that exposure of neutrophils to the *Bacillus* proteins induces chemotaxis and the activation of neutrophils via the IP_3 pathway. Activated neutrophils release elevated level of IL-6 and IL-1β which play a central role in inducing an inflammatory reaction in dermal tissue [43, 44]. The secretion of IL-1β and TNF-α is associated with the expression of NF-κB [55]. Azelaic acid, a therapy for the treatment of rosacea, has been shown to modulate the inflammatory response by suppressing IL-1β, TNF-α, and IL-6 secretion in normal human keratinocytes by activation of peroxisome-proliferator-activated receptor (PPAR)-γ along the NF-κB pathway [56].

The IP_3 pathway has been a pharmacological target as a treatment for diseases to regulate the immune response and reduce inflammation [57–60]. This pathway could represent a potential anti-inflammatory target to prevent the persistent erythema and flushing episodes which are a hallmark of rosacea.

The etiology of rosacea is unclear but may involve genetic, immunological, and environmental factors. A role for bacteria as contributory factors in the etiology of the

condition has been suggested [2, 3, 24–26]. Bacteria residing within *Demodex* mites may stimulate neutrophil infiltration and activation once released from dead mites residing in the pilosebaceous unit. The results presented here demonstrate how neutrophil activation and inflammation may be induced by exposure to *Bacillus*-derived proteins. Understanding the role and interaction of the various factors associated with the induction and persistence of rosacea [6, 8] should allow the development of more targeted therapies to reduce inflammation and ultimately cure this disfiguring condition.

ACKNOWLEDGMENTS

F. McM is funded by the Programme in BioAnalysis and Therapeutics (BioAT) by the Higher Education Authority of Ireland through PRTLI V.

Compliance with Ethical Standards

Conflict of interest. S. Paulie is employed by, and part-owner of, Mabtech AB. C. Smedman is employed by Mabtech AB. The other authors (F.McMahon, N. Banville, D. Bergin, E. Reeves, K. Kavanagh) have no conflicts of interest to declare.

REFERENCES

1. Powell, F.C. 2005. Rosacea. *New England Journal of Medicine* 352: 793–803.
2. Holmes, A.D. 2013. Potential role of microorganisms in the pathogenesis of rosacea. *Journal of American Academy of Dermatology* 69: 1025–32.
3. Jarmuda, S., N. O'Reilly, R. Zaba, O. Jakubowicz, A. Szkaradkiewicz, and K. Kavanagh. 2012. The potential role of *Demodex folliculorum* mites and bacteria in the induction of rosacea. *Journal of Medical Microbiology* 61: 1504–10.
4. Wilkin, J., M. Dahl, M. Detmar, L. Drake, A. Feinstein, R. Odom, and F. Powell. 2002. Standard classification of rosacea: Report of the National Rosacea Society Expert Committee on the Classification and Staging of Rosacea. *Journal of American Academy of Dermatology* 46: 584–87.
5. Crawford, G.H., M.T. Pelle, and W.D. James. 2004. Rosacea: I. Etiology, pathogenesis, and subtype classification. *Journal of American Academy of Dermatology* 51: 327–41.
6. Gupta, A.K., and M.M. Chaudhry. 2005. Rosacea and its management: an overview. *Journal of the European Academy of Dermatology and Venereology* 19: 273–85.
7. Pelle, M.T., G.H. Crawford, and W.D. James. 2004. Rosacea: II. Therapy. *Journal of American Academy of Dermatology* 51: 499–512.
8. Yamasaki, K., and R.L. Gallo. 2009. The molecular pathology of rosacea. *Journal of Dermatological Science* 55: 77–81.
9. Akamatsu, H., M. Oguchi, S. Nishijima, Y. Asada, M. Takashi, T. Ushijima, and Y. Niwa. 1990. The inhibition of free radical generation by human neutrophils through the synergistic effect of metronidazole with palmitoleic acid: a possible mechanism of metronidazole in rosacea and acne. *Archives of Dermatological Research* 282: 449–54.
10. Miyashi, Y., A. Yoshioka, S. Imamura, and Y. Niwa. 1986. Effect of antibiotics on the generation of reactive oxygen species. *Journal of Investigative Dermatology* 86: 449–53.
11. Yoshioka, A., Y. Miyachi, S. Imamura, and Y. Niwa. 1986. Antioxidant effects of retinoids on inflammatory skin diseases. *Archives of Dermatological Research* 278: 177–83.
12. Starkey, P.M., A.J. Barrett, and M.C. Burleigh. 1977. The degradation of articular collagen by neutrophil proteinases. *Biochimica et Biophysica Acta* 483: 386–97.
13. Berton, A., G. Godeau, H. Emonard, K. Baba, P. Bellon, W. Hornebeck, and G. Bellon. 2000. Analysis of the *ex vivo* specificity of human gelatinases A and B towards skin collagen and elastic fibers by computerized morphometry. *Matrix Biology* 19: 139–148.
14. Devaney, J.M., C.M. Greene, C.C. Taggart, T.P. Carroll, S.J. O'Neill, and N.G. McElvaney. 2003. Neutrophil elastase up-regulates interleukin-8 via toll-like receptor 4. *FEBS Letters* 544: 129–132.
15. Kuwahara, I., E.P. Lillehoj, W. Lu, I.S. Singh, Y. Isohama, T. Miyata, and K.C. Kim. 2006. Neutrophil elastase induces IL-8 gene transcription and protein release through p38/NF-κB activation via EGFR trans-activation in a lung epithelial cell line. *American Journal of Physiology. Lung Cellular and Molecular Physiology* 291: 407–416.
16. Tintinger, G., H.C. Steel, and R. Anderson. 2005. Taming the neutrophil: calcium clearance and influx mechanisms as novel targets for pharmacological control. *Clinical Experimental Immunology* 141: 191–200.
17. Bokoch, G.M. 1995. Chemoattractant signaling and leukocyte activation. *Blood* 86: 1649–1660.
18. Binah, O., M. Shilkrut, G. Yaniv, and S. Larisch. 2004. The Fas receptor 1,4,5-IP3 cascade: a potential target for treating heart failure and arrhythmias. *Annals of the New York Academy of Science* 1015: 338–350.
19. Wacker, M.J., L.M. Kosloski, W.J.R. Gilbert, C.D. Touchberry, D.S. Moore, J.K. Kelly, M. Brotto, and J.A. Orr. 2009. Inhibition of thromboxane A_2-induced arrhythmias and intracellular calcium changes in cardiac myocytes by blockade of the inositol trisphosphate pathway. *Journal of Pharmacological and Experimental Therapeutics* 331: 917–924.
20. Vance, J. 1986. Demodicidosis – Do *Demodex* mites cause Disease? *Current Concepts in Skin Disorder* 10–18.
21. Bonner, E., P. Eustace, and F.C. Powell. 1993. The *Demodex* mite population in Rosacea. *Journal of American Academy of Dermatology* 28: 443–448.
22. Erbağci, Z., and O. Ozgöztaşi. 1998. The significance of *Demodex folliculorum* density in rosacea. *International Journal of Dermatology* 37: 421–425.
23. Ni Raghallaigh, S., K. Bender, N. Lacey, L. Brennan, and F.C. Powell. 2012. The fatty acid profile of the skin surface lipid layer in papulopustular rosacea. *British Journal of Dermatology* 166: 279–287.
24. Dahl, M.V., A.J. Ross, and P.M. Schlievert. 2004. Temperature regulates bacterial protein production: possible role in rosacea. *Journal of American Academy of Dermatology* 50: 266–272.
25. Lacey, N., S. Delaney, K. Kavanagh, and F.C. Powell. 2007. Mite-related bacterial antigens stimulate inflammatory cells in rosacea. *British Journal of Dermatology* 157: 474–481.
26. Whitfeld, M., N. Gunasingam, L. Joo Leow, K. Shirato, and V. Preda. 2011. *Staphylococcus epidermidis*: a possible role in the pustules of rosacea. *Journal of American Academy of Dermatology* 64: 49–52.
27. Kuhnigk, T., E.M. Borst, A. Breunig, H. König, M.D. Collins, R.A. Hutson, and P. Kämpfer. 1995. *Bacillus oleronius* sp. nov., a member

of the hind-gut flora of the termite *Reticulitermes santonensis* (Feytaud). *Canadian Journal of Microbiology* 41: 699–706.
28. Szkaradkiewicz, A., I. Chudzicka-Strugała, T.M. Karpiński, O. Goślińska-Pawłowska, T. Tułecka, W. Chudzicki, A.K. Szkaradkiewicz, and R. Zaba. 2012. *Bacillus oleronius* and *Demodex* mite infestation in patients with chronic blepharitis. *Clinical Microbiology and Infection* 18: 1020–1025.
29. Li, J., N. O'Reilly, H. Sheha, R. Katz, V.K. Raju, K. Kavanagh, and S.C. Tseng. 2010. Correlation between ocular *Demodex* infestation and serum immunoreactivity to *Bacillus* microbial proteins in patients with facial rosacea. *Ophthalmology* 117: 870–877.
30. O'Reilly, N., N. Menezes, and K. Kavanagh. 2012. Positive correlation between serum immuno-reactivity to *Demodex*-associated *Bacillus* proteins and erythematotelangiectic rosacea. *British Journal of Dermatology* 167: 1032–1036.
31. Jarmuda, S., F. McMahon, R. Zaba, N. O'Reilly, O. Jakubowicz, A. Holland, A. Szkaradkiewicz, and K. Kavanagh. 2014. Correlation between serum reactivity to *Demodex*-associated *Bacillus oleronius* proteins, and altered sebum levels and *Demodex* populations in erythematotelangiectatic rosacea patients. *Journal of Medical Microbiology* 63: 258–262.
32. O'Reilly, N., C. Gallagher, K.R. Katikireddy, M. Clynes, F. O'Sullivan, and K. Kavanagh. 2012. *Demodex*-associated *Bacillus* proteins induce an aberrant wound healing response in a corneal epithelial cell line (hTCEpi): possible implications for corneal ulcer formation in ocular rosacea. *Investigative Ophthalmology and Visual Science* 53: 3250–3259.
33. McMahon, F.W., C. Gallagher, N. O'Reilly, M. Clynes, F. O'Sullivan, and K. Kavanagh. 2014. Exposure of a corneal epithelial cell line (hTCEpi) to *Demodex*-associated *Bacillus* proteins results in an inflammatory response. *Investigative Ophthalmology and Visual Science* 55: 7019–7028.
34. O'Reilly, N., D. Bergin, E.P. Reeves, N.G. McElvaney, and K. Kavanagh. 2012. *Demodex*-associated bacterial proteins induce neutrophil activation. *British Journal of Dermatology* 166: 753–760.
35. Reeves, E.P., H. Lu, H.L. Jacobs, C.G. Messina, S. Bolsover, G. Gabella, E.O. Potma, A. Warley, J. Roes, and A.W. Segal. 2002. Killing activity of neutrophils is mediated through activation of proteases by K+ flux. *Nature* 416: 291–297.
36. Eichner, R.D., M. Al Salami, P.R. Wood, and A. Müllbacher. 1986. The effect of gliotoxin upon macrophage function. *International Journal of Immunopharmacology* 8: 789–797.
37. Jayachandran, R., V. Sundaramurthy, B. Combaluzier, P. Mueller, H. Korf, K. Huygen, T. Miyazaki, I. Albrecht, J. Massner, and J. Pieters. 2007. Survival of mycobacteria in macrophages is mediated by coronin 1-dependent activation of calcineurin. *Cell* 130: 37–50.
38. Rossi, F. 1986. The O_2-forming NADPH oxidase of the phagocytes: Nature, mechanisms of activation and function. *Biochimica et Biophysica Acta* 853: 65–89.
39. Lew, P.D. 1990. Receptors and intracellular signaling in human neutrophils. *The American Review of Respiratory Disease* 141: S127–131.
40. Billah, M.M. 1993. Phospholipase D and cell signaling. *Current Opinions in Immunology* 5: 114–123.
41. Thelen, M., and U. Wirthmueller. 1994. Phospholipases and protein kinases during phagocyte activation. *Current Opinions in Immunology* 6: 106–112.
42. Pollard, T.D., and J.A. Cooper. 2009. Actin, a central player in cell shape and movement. *Science* 326: 1208–1212.
43. Brink, N., M. Szamel, A.R. Young, K.P. Wittern, and J. Bergemann. 2000. Comparative quantification of IL-1beta, IL-10, IL-10r, TNFalpha and IL-7 mRNA levels in UV-irradiated human skin *in vivo*. *Inflammation Research* 49: 290–296.
44. Grossman, R.M., J. Krueger, D. Yourish, A. Granelli-Piperno, D.P. Murphy, L.T. May, T.S. Kupper, P.B. Sehgal, and A.B. Gottlieb. 1989. Interleukin 6 is expressed in high levels in psoriatic skin and stimulates proliferation of cultured human keratinocytes. *Proceedings of the National Academy of Science of the USA* 86: 6367–6371.
45. Aylesworth, R., and J.C. Vance. 1982. *Demodex folliculorum* and *Demodex brevis* in cutaneous biopsies. *Journal of American Academy of Dermatology* 7: 583–589.
46. Akilov, O.E., and K.Y. Mumcuoglu. 2003. Association between human demodicosis and HLA class I. *Clinical and Experimental Dermatology* 28: 70–73.
47. Casas, C., C. Paul, M. Lahfa, B. Livideanu, O. Lejeune, S. Alvarez-Georges, C. Saint-Martory, A. Degouy, V. Mengeaud, H. Ginisty, E. Durbise, A.M. Schmitt, and D. Redoulès. 2012. Quantification of *Demodex folliculorum* by PCR in rosacea and its relationship to skin innate immune activation. *Experimental Dermatology* 21: 906–910.
48. Grice, E.A., and J.A. Segre. 2011. The skin microbiome. *Nature Reviews Microbiology* 9: 244–253.
49. Leyden, J.J., K.J. McGinley, O.H. Mills, and A.M. Kligman. 1975. Propionibacterium levels in patients with and without acne vulgaris. *Journal of Investigative Dermatology* 65: 382–384.
50. Somerville, D.A. 1969. The normal flora of the skin in different age groups. *British Journal of Dermatology* 81: 248–258.
51. Murillo, N., J. Aubert, and D. Raoult. 2014. Microbiota of Demodex mites from rosacea patients and controls. *Microbial Pathogenesis* 71–72: 37–40.
52. Putney, J.W. 2007. Recent breakthroughs in the molecular mechanism of capacitative calcium entry (with thoughts on how we got here). *Cell Calcium* 42: 103–110.
53. Berridge, M.J. 1995. Capacitative calcium entry. *Biochemistry Journal* 312: 1–11.
54. Taylor, C.W., P.C. da Fonseca, and E.P. Morris. 2004. IP3 receptors: the search for structure. *Trends in Biochemical Science* 29: 210–219.
55. Lawrence, T. 2009. The nuclear factor NF-κB pathway in inflammation. *Cold Spring Harbor Perspectives in Biology* 1(6): a001651.
56. Mastrofrancesco, A., M. Ottaviani, N. Aspite, G. Cardinali, E. Izzo, K. Graupe, C.C. Zouboulis, E. Camera, and M. Picardo. 2010. Azelaic acid modulates the inflammatory response in normal human keratinocytes through PPARγ activation. *Experimental Dermatology* 19: 813–820.
57. Eishingdrelo, H., and S. Kongsamut. 2013. Minireview: targeting GPCR activated ERK pathways for drug discovery. *Current Chemical Genomics and Translational Medicine* 7: 9–15.
58. Brown, K.M., and D.K. Tracy. 2013. Lithium: the pharmacodynamic actions of the amazing ion. *Therapeutic Advances in Psychopharmacology* 3: 163–176.
59. Kliem, C., A. Merling, M. Giaisi, R. Köhler, P.H. Krammer, and M. Li-Weber. 2012. Curcumin suppresses T cell activation by blocking Ca2+ mobilization and nuclear factor of activated T cells (NFAT) activation. *Journal of Biological Chemistry* 287: 10200–10209.
60. Galeotti, N., A. Bartolini, M. Calvani, R. Nicolai, and C. Ghelardini. 2004. Acetyl-L-carnitine requires phospholipase C-IP3 pathway activation to induce antinociception. *Neuropharmacology* 47: 286–294.