Fish and Shellfish Immunology Reports 5 (2023) 100116

Contents lists available at ScienceDirect

Fish and Shellfish Immunology Reports

journal homepage: www.sciencedirect.com/journal/fish-and-shellfish-immunology-reports

# The effect of PACAP administration on LPS-induced cytokine expression in the Atlantic salmon SHK-1 cell line.

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ARTICLE INFO

*Keywords:*
Atlantic salmon
PACAP
innate immunity
immunostimulant
SHK-1
*Flavobacterium columnare*
cytokines

ABSTRACT

Recent work has identified pituitary adenylate cyclase activating polypeptide (PACAP) as a potential antimicrobial and immune stimulating agent which may be suitable for use in aquaculture. However, its effects on teleost immunity are not well studied and may be significantly different than what has been observed in mammals. In this study we examined the effects of PACAP on the Atlantic salmon macrophage cell line SHK-1. PACAP was able to increase the expression of LPS-induced il-1β in at concentrations of 1 uM when administered 24h prior to LPS stimulation. Furthermore, concentrations as low as 40nM had an effect when administered both 24h prior and in tandem with LPS. PACAP was also capable of increasing the expression of il-1β and tnf-α in SHK-1 cells challenged with a low dose of heat-killed *Flavobacterium columnare*. We attempted to get a better understanding of the mechanism underlying this enhancement of il-1β expression by manipulating downstream signaling of PACAP with inhibitors of phosphodiesterase and phospholipase C activity. We found that inducing cAMP accumulation with phosphodiesterase inhibitors failed to recapitulate the effect of PACAP administration on LPS-mediated il-1β expression by PACAP, while use of a phospholipase C inhibitor caused a PACAP-like enhancement in LPS-mediated il-1β expression. Interestingly, the VPAC1 receptor inhibitor PG97-269, but not the PAC1 inhibitor max.d.4, also was capable of causing a PACAP-like enhancement in LPS-mediated il-1β expression. This suggests that fish do not utilize the PACAP receptors in the same manner as mammals, but that it still exerts an immunostimulatory effect that make it a good immunostimulant for use in aquaculture.

## Introduction

Pituitary adenylate cyclase-activating polypeptide is an extracellular signaling molecule of the secretin-glucagon super family. It has two active forms: one 27- and one 38-amino acid form both derived from cleavage of a 176-residue precursor. PACAP has high sequence homology with another signaling molecule of the same class, vasoactive intestinal peptide [1]. PACAP signaling is achieved through activation of g-protein coupled receptors (GPCRs), which in turn generate a variety of intracellular second messengers like cyclic AMP, diacyl glycerol and inositol triphosphate. Three GPCRs are known to be activated by PACAP: PAC1, for which PACAP is the exclusive ligand, and the receptors VPAC1 and 2, which can also be activated by vasoactive intestinal peptide [1]. Originally discovered as a stimulator of adenylate cyclase in pituitary cells, PACAP is now known to participate in a wide range of physiological functions, including growth, glucocorticoid hormone secretion during stress and the immune response [1,2].

PACAP has a variety of interesting properties when it comes to its role in the immune system. While nominally a signaling molecule, it has been shown that PACAP can function as an antimicrobial peptide. This antimicrobial activity has been shown to be present for both fish and mammalian PACAP variants against a range of bacterial and fungal pathogens [3–7]. In mice, for example, PACAP was shown to be upregulated in response to bacterial infection and was able to inhibit the

*Abbreviations:* cAMP, cyclic adenosine monophosphate; CD, cluster of differentiation; GPCR, g-protein coupled receptor; IBMX, 3-isobutyl-1-methylxanthine; il-1β, interleukin 1-beta; il-10, interleukin ten; HSC, heat shock cognate; LPS, lipopolysaccharide; PACAP, pituitary adenylate cyclate activating peptide; PG97, PG97-269; PLC, phospholipase C; SHK, salmon head kidney; tnf-α, tumour necrosis factor alpha; VIP, vasoactive intestinal peptide.

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https://doi.org/10.1016/j.fsirep.2023.100116
Received 18 August 2023; Received in revised form 13 September 2023; Accepted 16 September 2023
Available online 17 September 2023

growth of *Staphylococcus aureus, Escherichia coli* and *Candida albicans* in media simulating conditions in brain and abscesses. In tandem with these antimicrobial effects PACAP has an impact on host immune signaling as well. In mammals PACAP has been described to have immune modulatory properties that generally inhibit inflammation. This includes both suppression of inflammatory cytokine secretion of macrophages and dendritic cells as well as affecting CD4+ T-cell stimulation and polarization [8,9]. It has been speculated that these two roles of PACAP complement each other particularly well in the context of nervous tissue, with the antimicrobial activity directly inhibiting pathogen growth and the immune signaling aspect preventing damaging levels of inflammation [4].

Research also shows that PACAP is involved in teleost immune function, but interestingly the evidence tends to suggest that it acts to stimulate innate and adaptive immunity. In grass carp, for example, PACAP moderately increased pro-inflammatory cytokine expression in head kidney leucocytes stimulated with lipopolysaccharide [10]. In *Clarias gariepinus* and *Orechromis niloticus* PACAP has been shown to increase the concentration of serum lysozyme, IgM and NOS metabolites [5,11]. PACAP treatment resulted in an increase in tnf-α expression in the gut of *Paralichthys olivaceus*) [12]. During a study on the direct antimicrobial effects of PACAP we observed that treatment with concentrations of PACAP much lower than what is required for direct antimicrobial action could also reduce the survival of the fish pathogen *Flavobacterium psychrophilum* survival when co-cultured with the rainbow trout macrophage cell line RTS-11[13]. We also observed that PACAP alone could induce a small increase in il-1β expression, which is suggestive of an immune stimulatory effect [13]. Further work has shown that PACAP could induce upregulation of antiviral cytokine gene expression and enhance the survival of rainbow trout fry challenged with viral haemorrhagic septicaemia virus and that PACAP treatment alters the expression of toll-like receptor pathway genes in channel catfish [14,15].

The global finfish aquaculture industry produced 57.5 million tonnes of product in 2022 worth 146.1 billion US dollars, with over 50% used for human consumption [16]. Disease is a significant source of economic loss in this industry. For example, in 2022 Norway reported 16% of Atlantic salmon in the saltwater growth phase and while a % loss cannot be calculated, over 35 million salmon over 3gm were lost in the freshwater growth phase that year [17]. These clearly represent a significant economic loss in a critical food industry. While fish are vaccinated, these vaccines are not as effective as they could be due to a variety of reasons [18–20]. Thus, it is common practice to treat potential disease with antibiotic enhanced feed. Indeed in 2021, the Chilean aquaculture industry alone used 450 tons of antibiotics [20]. Given that fish do not eat all the feed and excrete excess antibiotics, this can lead to antibiotic resistance in both fish and human pathogens. Thus, effective alternatives to antibiotics that do not lead to resistance and break down quickly in the environment are desperately needed and PACAP represents a promising antibiotic alternative.

While relatively well studied in mammals, little work has been done to characterize the mechanisms underlying PACAP's immune-related effects in teleosts. To this end, we conducted a study examining if PACAP could enhance pathogen associate molecular pattern (PAMP)-mediated cytokine changes in salmonid macrophages, and then determining how these changes were affected by manipulating various effectors of PACAP signaling. We used the *Salmo salar* SHK-1 cell line, a macrophage-like line derived from head kidney tissue, as this cell-line had previously been shown to be responsive to stimulation with lipopolysaccharide (LPS), which we wanted to use as an immune stimulant [21].

## Methods

### *Peptides*

A peptide based on the *Clarias gariepinus* PACAP-38 with an amino acid sequence of HSDGIFTDSYSRYRKQMAVKKYLAAVLGRRYRQR FRNK was chemically synthesized and purchased from Biomatik. An inert "control" peptide based on the sequence of *Salmo salar* heat shock cognate protein 70, having the sequence PGAGGAAPGGGGSS, was synthesized by and ordered from Biomatik. Upon reception, peptides were dissolved in PBS at a concentration of 1mM and aliquoted and stored at -20°C. The peptide VPAC1 and PAC1 receptor inhibitors were ordered from Sigma, and dissolved in PBS at a concentration of 1mM then aliquoted and stored at -20°C.

### *Cell maintenance*

The *Salmo salar* macrophage-like cell line SHK-1 was generously provided by Steven Lord of the University of Guelph. Cells were grown in Leibovitz's L-15 medium (Cytivia Life Sciences) supplemented with 10% FBS (Gibco) and Gibco Antibiotic-Antimycotic containing 100 μg/ml of penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. Cells were passaged weekly. Cells were detached using TrypLE Express reagent (Thermo-Fisher) then resuspended in fresh media and divided equally between new flasks.

### *Immune stimulation experiments*

LPS (Sigma) was dissolved in sterile PBS to a final concentration of 5mg/ml and stored at -20°C as single use aliquots. Safingol (Sigma) and IBMX (Sigma) were dissolved in sterile dimethylsulphoxide (Sigma) and stored at -20°C prior to use. Heat killed *F. columnare* was prepared by overnight culture in cytophaga broth, after which the optical density was measured, and samples were spread on cytophaga agar plates for quantification. Cells were collected by centrifugation (10,000xG) and resuspended in PBS at a concentration of x CFU/ml. They were rendered heat inactivated by immersion in 95°C water for half an hour, with verification of inactivation assessed by plating on cytophaga agar plates [22].

Cells were split into six well plates and left to attach overnight in L-15 media supplemented with 10% fetal bovine serum and Gibco Antibiotic-Antimycotic. Media was then replaced with unsupplemented L-15 24 h prior to the immune stimulation trial. In experiments with PACAP pretreatments, either PACAP or (HSC) peptide was added immediately after replacing the media with unsupplemented L-15 24 h prior to the immune stimulating stage. After stimulation cells were harvested by scraping and stored at -80°C until RNA extraction.

### *qRT-PCR Reactions*

RNA extraction was performed using Qiagen RNeasy kits (Qiagen) as per the manufacturer's instructions. An on-column DNA digestion step was performed to ensure samples were free of genomic DNA. Complementary DNA was generated from isolated RNA using qScript cDNA supermix as per the manufacturer's instructions. 250ng of RNA was used per cDNA synthesis reaction.

Relative transcript levels of ef-1α, il-1β, il-10, pac1, vpac1 and tnf-α were determined using qRT-PCR. The primers used are summarized in Table 1 [6,21,23]:

PCR reactions were prepared using Wisent Advanced qPCR mix with supergreen dye (Wisent). Each 10μl reaction was run in duplicate and contained 1μl primer mix (800nM), 1 μl of water, 5μl of qPCR mix and 3μl of template DNA. The qRT-PCR reactions consisted of a ten-minute 95°C pre-incubation step followed by forty cycles of denaturation for 10 s at 95°C, annealing for 5 s at 60°C and extension for 8 s at 72°C. A melting curve from 65°C to 97°C with reads every 5 s was performed per run to validate product specificity. All qPCR data was analyzed using the ΔΔCt method with data normalized to expression of ef-1α, with data converted and presented as log-2 fold change relative to ef-1α. Data was averaged across four experimental replicates. Statistical analysis was performed using GraphPad Prism 9 software. Significance was assessed by one-way ANOVA and Tukey's *post-hoc* test.

**Table 1**
Primers used in qPCR experiments, with source accession numbers (F: forward, R: reverse)

| Gene | Primers | Accession number |
|---|---|---|
| ef-1α | **F:** CGCACAGTAACACCGAAACGAATTAAGC<br>**R:** GCCTCCGCACTTGTAGATCAGATG | XM_014141923.2 |
| il-1β | **F:** CCACAAAGTGCATTTGAAC<br>**R:** GCAACCTCCTCTAGGTGC | NM_001123582.1 |
| il-10 | **F:** GGGTGTCACGCTATGGACAG<br>**R:** TGTTTCCGATGGAGTCGATG | EF165028.1 |
| pac1 | **F:** ATGCTGATTTAAGCCTCCACCA<br>**R:** GTGGAGGTGAGCTCTTGAGG | HG000282 |
| vpac1 | **F:** GTCCAACCAGTACTTGAGGCTG<br>**R:** CAACCGCAAATCCCTGGAATG | HG000283 |
| tnf-α | **F:** CGCGAGCATACCACTCCTCT<br>**R:** TCGGACTCAGCATCACCGTA | DQ787157.1 |

## Results

### *Effect of PACAP on LPS-induced il-1β and tnf-α expression*

Our first objective was to determine if PACAP could affect inflammatory cytokine expression induced by bacteria derived PAMPs in SHK-1 cells. A peptide derived from the Atlantic salmon heat shock protein 70 cognate (HSC) was co-administered with LPS-only treatments to control for possible non-specific effects resulting from addition of synthetic peptides to the media. We first tested the effect of a twelve-hour pre-treatment with PACAP on il-1β and tnf-α expression induced by four-hour LPS (15ug/ml) exposure (Fig. 1A and B). PACAP had no statistically significant effect on tnf-α expression at any of the concentrations tested, however il-1β expression was significantly higher in cells treated with both LPS and 1000nM PACAP than those treated with LPS and the HSC peptide (1000nM). Next, we altered the experiment to include a second dose or "pulse" of PACAP administered when the cells were exposed to LPS in order to determine if the effect of PACAP on il-1β expression could be further enhanced (Fig. 1C and D). Cells treated with two pulses of PACAP at concentrations of 40, 600 and 1000nM all had higher levels of LPS-induced il-1β expression than that of LPS treated with two pulses of HSC peptide (1000nM). Compared to the single dose experiment adding a second pulse resulted in concentrations of 40 and 600nM being sufficient to increase il-1β expression above that of the LPS control. This is consistent with earlier work where sustained administration of PACAP was used to achieve antiviral effects in rainbow trout [15].

### *Effect of PACAP on il-1β and tnf-α expression induced by heat-killed Flavobacterium columnare*

Next, we wanted to determine if this effect occurs in response to a crude mix of PAMPs derived from whole bacteria rather than purified LPS. To this end we investigated the effect of PACAP administration on cytokine expression induced by the Gram-negative fish pathogen *Flavobacterium columnare*. We used the same experimental conditions as the LPS trials with pulsed PACAP administration but used heat-killed bacteria in the place of LPS. We used two concentrations of heat killed bacteria, the first ("High" Fig. 2A and B) estimated to be equivalent to a MOI of 1.2 and the second ("Low" Fig. 2C and D) estimated to be equivalent to a MOI of 0.4. The high concentration of heat killed bacteria elicited a vigorous inflammatory response in the SHK-1 cells compared to that of LPS in terms of average il-1β and tnf-α mRNA levels. However, there was no statistically significant difference in il-1β expression between cells treated with and without PACAP with this concentration of heat killed bacteria. Likewise, PACAP had no effect on tnf-α expression, although there was high variability of tnf-α expression in samples treated with higher concentrations of PACAP (Fig. 2B). When exposed to the lower concentration of heat-killed bacteria, SHK-1 cells showed a more modest increase in il-1β and tnf-α expression compared to the high concentration of heat-killed bacteria. Under these conditions, il-1β expression was elevated in cells stimulated with heat-killed bacteria and treated with 200nM PACAP compared to cells treated with heat-killed bacteria and the HSC peptide. Interestingly, tnf-α expression was also

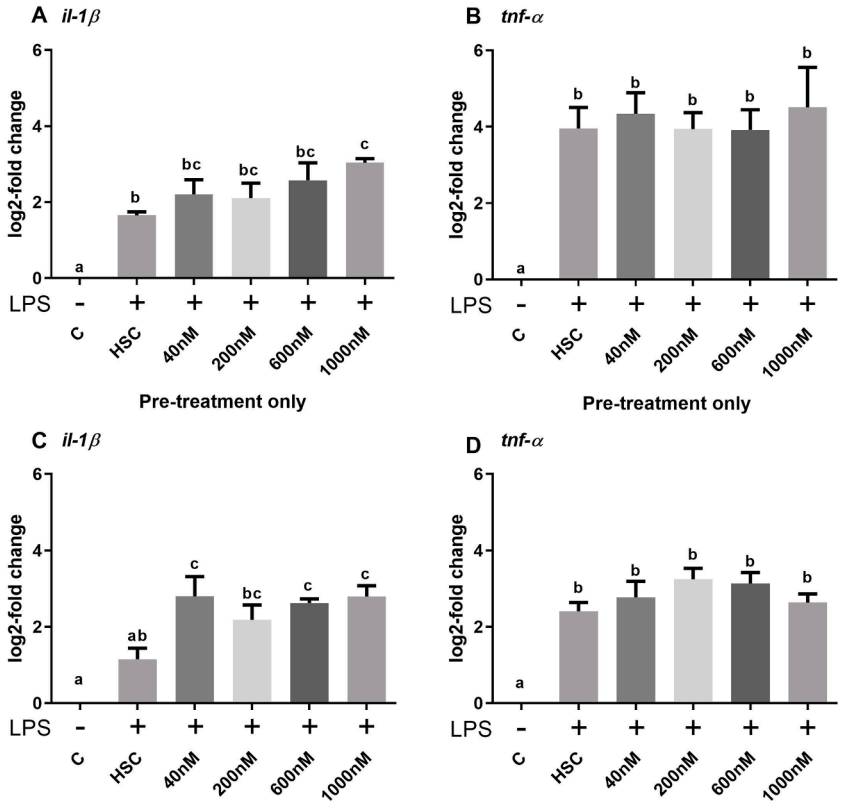


**Fig. 1.** Two experiments were conducted: in the first, cells were treated with varying concentrations of PACAP (40, 200, 600 and 1000nM) or a synthetic peptide derived from heat shock cognate protein 70 (HSC) 24 h prior to stimulation with LPS with resulting changes in il-1β (A) and tnf-α (B) expression measured. In the second, the same experiment was conducted but an additional pulse of PACAP or control peptide were added prior to stimulation with changes in il-1β (C) and tnf-α (D) expression measured. Data in panels are derived from four independent experiments presented as mean log2 fold change values with standard error of the mean indicated by vertical bars. Lowercase letters denote significant differences at $p < 0.05$.

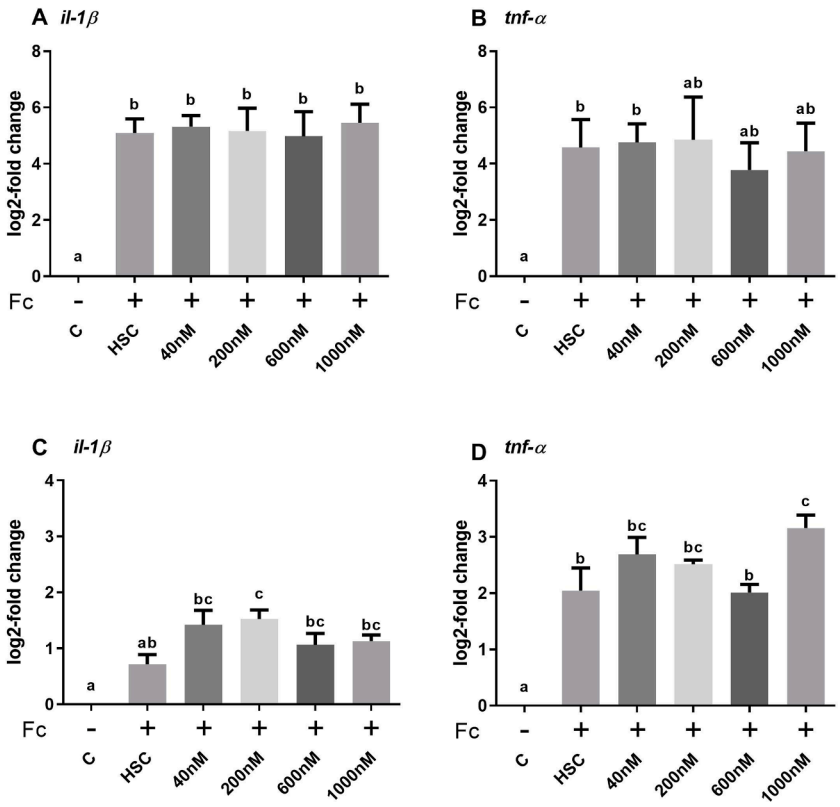


**Fig. 2.** Effect of PACAP exposure on il-1β and tnf-α expression in SHK-1 cells induced by heat killed *Flavobacterium columnare*. Two experiments were again conducted: in the first, cells were treated with varying concentrations of PACAP (40, 200, 600 and 1000 nM) or a synthetic peptide derived from heat shock cognate protein 70 (HSC) 24 h prior to stimulation with a high concentration of heat killed bacteria (estimated equivalent MOI of 1.2) with resulting changes in il-1β (A) and tnf-α (B) expression measured. In the second, the same experiment was conducted but a lower concentration of heat killed bacteria (estimated equivalent MOI of 0.4) was used for stimulation with subsequent changes in il-1β (C) and tnf-α (D) expression being measured. Data in panels are derived from four independent experiments presented as mean log2 fold change values with standard error of the mean indicated by vertical bars. Lowercase letters denote significant differences at $p < 0.05$.

elevated in stimulated cells treated with 1000nM PACAP compared to those treated with the control peptide (Fig. 2D).

*The effect of phosphodiesterase inhibition on cytokine expression is distinct from that of PACAP administration*

Degradation of cAMP *in vivo* is catalyzed by a family of enzymes known as phosphodiesterases (PDEs), and phosphodiesterase inhibition is commonly used in experimental settings to induce intracellular cAMP accumulation [24]. We used this method as an approximate way of measuring the effect of cAMP accumulation on LPS-induced il-1β, tnf-α and il-10 expression in SHK-1 cells, both in tandem with and independent of PACAP administration. To achieve PDE inhibition we used the broad-spectrum inhibitor 3-isobutyl-1-methylxanthine (IBMX). In these experiments IBMX was added at a concentration of 100uM along with either PACAP or HSC 24h and again immediately prior to stimulation with LPS for 4h. We also used an elevated LPS concentration (30 μg/ml) to increase the degree of cytokine expression change and provide more scope for a decrease should IBMX have an anti-inflammatory effect. The results from these experiments are shown in Fig. 3. Treatment with IBMX alone resulted in a statistically significant decrease in il-1β expression (Fig. 3A). In LPS stimulated cells, IBMX treatment did not alter il-1β expression levels by itself, however the PACAP-mediated increase in il-1β expression seen previously was not present in cells treated with IBMX (Fig. 3A). Expression of tnf-α in stimulated cells treated with IBMX was lower than in cells stimulated with LPS and treated with 1000nM PACAP (Fig. 3B). With regards to il-10 there was a decrease in expression associated with IBMX administration. Furthermore, we observed that this level of LPS stimulation induced a detectable increase in the level of il-10 expression when added with either control peptide or PACAP peptide- this increase was absent however when cells were treated with IBMX (Fig. 3C). IBMX administration caused a significant increase in expression of the vpac1 receptor, but not pac1 (Fig. 3D and E). We were unable to detect vpac2 in this cell line (data not shown). This is consistent with previous results showing an absence of vpac2 expression in rainbow trout cultured macrophages and peripheral blood mononuclear cells [25]

*Administration of safingol affects il-1β expression in a manner similar to PACAP*

In addition to exploring aspects of cAMP-mediated signaling we also wanted to examine the effect of disrupting PACAP signaling through the phospholipase C (PLC) pathway. For this purpose, we used safingol, a sphinogsine-derived inhibitor of various protein kinase C family members which are downstream effectors of PLC signaling. As with other experiments safingol (40 μg/ml) was added concurrently with the PACAP and HSC peptide treatments 24 h and immediately prior to LPS administration. Safingol treatment in combination with LPS resulted in elevated levels of il-1β expression (Fig. 4A). This level of il-1β expression was comparable to that found in cells treated with both LPS and 1000 nM PACAP (Fig. 4). Treatment of LPS-stimulated cells with safingol and PACAP together did not raise levels of il-1β expression beyond that which was observed by treating LPS-stimulated cells with either safingol or PACAP by themselves (Fig. 4A). Addition of safingol to unstimulated cells did not induce significant changes il-1β expression (Fig. 4A). Safingol added to unstimulated cells was associated with a small increase in tnf-α expression, however safingol did not appear to affect tnf-α expression in cells exposed to LPS (Fig. 4B). Safingol treatment was also associated with an increase in il-10 expression (Fig. 4C). This increase in il-10 expression was observed in cells treated with safingol only, with LPS and safingol together, and with PACAP, LPS and safingol. There was no statistically significant difference in il-10 expression between safingol-treated groups. Both vpac1 and pac1 expression were unaffected by safingol treatment (Fig. 4D & C).

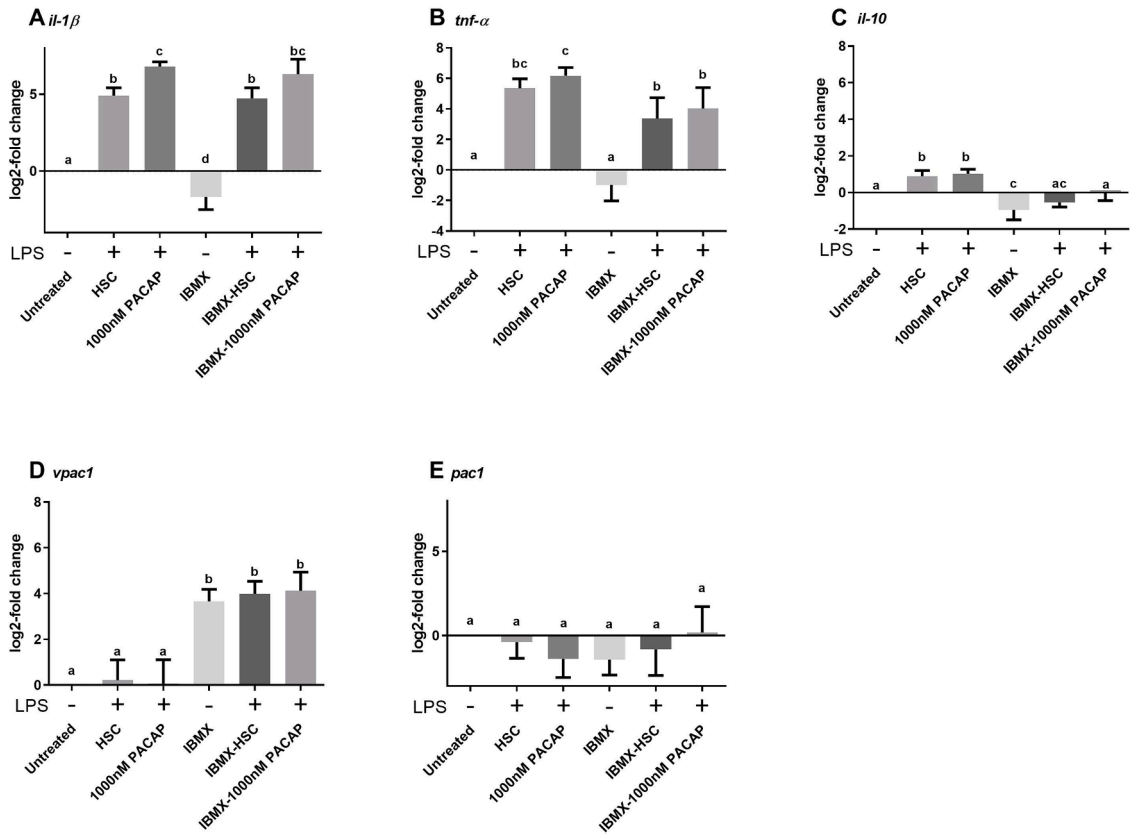


**Fig. 3.** Effect of phosphodiesterase inhibitor IBMX on LPS-induced il-1β (A), tnf-α (B), il-10 (C), vpac1 (D) and pac1 (E) expression in SHK-1 cells. Cells were treated cells were treated with 1000nM PACAP (PACAP), a 1000nM of HSC peptide (HSC) and 100 μM IBMX (IBMX) 24h and immediately prior to a 4h stimulation with 30 μg/ml LPS. Differences in levels of il-1β, tnf-α and il-10 transcripts as determined by qPCR are presented on the graphs. Data in panels are derived from four independent experiments presented as mean log2 fold change values with standard error of the mean indicated by vertical bars. Lowercase letters denote significant differences at $p < 0.05$.

*The VPAC-1 inhibitor PG97-269 was associated with increased LPS-induced il-1β expression*

Next, we wanted to ascertain what effect disrupting physiologically normal signaling of PACAP receptors would have on cytokine expression during LPS exposure. We therefore conducted experiments where we treated cells with PACAP receptor inhibitors prior to stimulation with LPS. Two inhibitors were used in these experiments: PG97-269 (PG97), a VPAC1 receptor inhibitor, and max.d.4 (md4), an inhibitor of the PAC1 receptor (Gourlet et al, 1997; Tatsuno et al, 2001). LPS-stimulated cells treated with 1000nM PG97 had higher levels of il-1β expression compared to those treated with 1000nM of the control peptide (Fig. 5A). Treatment with LPS and 1000nM PG97 also resulted in a detectable increase in il-10 expression whereas treatment with LPS alone did not (Fig. 5C). We did not detect any other statistically significant changes in il-1β, tnf-α or il-10 expression induced by LPS as a result of treating cells with these inhibitors. Treatment with the inhibitors did not alter levels of vpac1 and pac1 expression (Fig. 5D and E). We also attempted to inhibit PACAP-mediated changes to LPS-induced cytokine expression using these inhibitors, however we observed no effect on cytokine expression when added in equal-molar concentrations (data not shown).

## Discussion

The purpose of this study was twofold - first, to see if PACAP had an effect on LPS-induced inflammatory cytokine expression in salmon macrophages, and second to examine the contribution of various effectors of PACAP signalling to this effect. We found that treatment with PACAP increased LPS-induced expression of il-1β and that repeated administration of PACAP lowered the concentration necessary to achieve this effect. We did not see any statistically significant effect of PACAP treatment on LPS-induced tnf-α expression, however levels of both tnf-α and il-1β expression induced by treatment with low concentrations of heat-killed bacteria were increased by PACAP administration. We found that administration of the phosphodiesterase inhibitor IBMX did not produce similar effects on LPS-induced cytokine expression as PACAP and was by itself able to decrease il-1β expression. In contrast, treatment with the phospholipase C inhibitor safingol resulted in effects similar to PACAP with regards to LPS-induced il-1β expression, however safingol treatment also was associated with an increase in il-10 expression. Finally, the VPAC-1 inhibitor PG97-269 elicited an increase in LPS-induced il-1β expression when administered to SHK-1 cells.

Intracellular cAMP, through its actions on CREB and NFκβ, is generally understood to promote anti-inflammatory effects in mammalian macrophages [26–28]. While we did not directly measure levels of cAMP, our results suggest that the immune stimulatory effect associated with PACAP treatment in fish is unlikely to be due to PACAP induced cAMP accumulation. Treatment with IBMX strongly upregulated the expression of VPAC1 receptors, suggesting it had an impact on PACAP mediated signaling, however its effects on cytokine expression were not at all similar to those of PACAP treatment. Research into the effects of phosphodiesterase inhibition on immune function in teleosts is limited, but this very tentative evidence indicates that it has an effect similar to what is observed in mammalian macrophages [29,30]. Other work has shown that there may be an inverse relationship between cAMP accumulation and proinflammatory signaling in teleosts, with cAMP accumulation in gonads being inhibited by tnf-α and il-1β [31].

Our results showed that safingol treatment increased il-1β expression

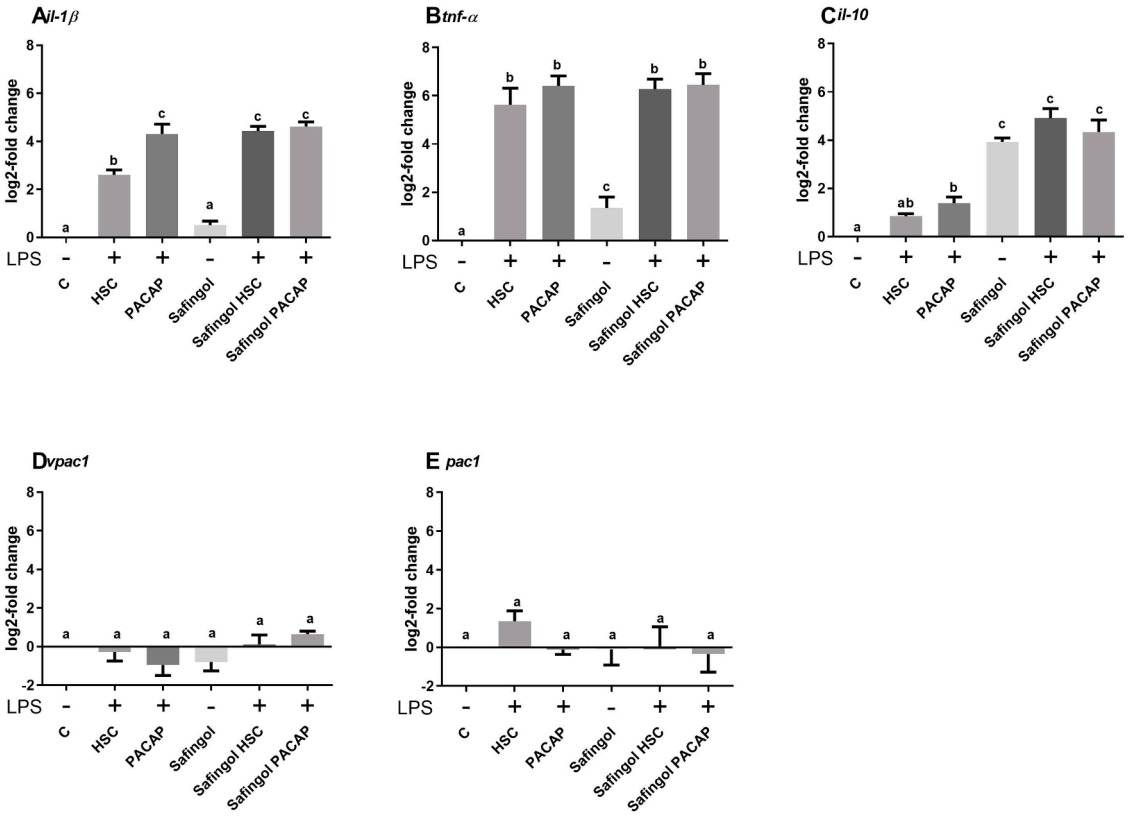


**Fig. 4.** Effect of PKC inhibitor safingol on LPS-induced il-1β (A), tnf-α (B), il-10 (C), vpac1 (D) and pac1 (E) expression in SHK-1 cells. Cells were treated cells were treated with 1000nM PACAP (PACAP), a 1000nM of HSC peptide (HSC) and 40 μM safingol (safingol) 24h and immediately prior to a 4h stimulation with 30 μg/ml LPS. Differences in levels of il-1β, tnf-α and il-10 transcripts as determined by qPCR are presented on the graphs. Data in panels are derived from four independent experiments presented as mean log2 fold change values with standard error of the mean indicated by vertical bars. Lowercase letters denote significant differences at $p < 0.05$.

induced by LPS in a manner strikingly similar to PACAP. More generally however the response to safingol treatment was distinct from PACAP in that it also elicited an increase in il-10 expression. In mammals, the role played by PKC signaling in innate immune activation is complex in part due to the involvement of multiple members of PKC protein family. Different PKC isoforms are associated with different toll-like receptors and are involved at multiple steps in the toll-like receptor activation pathways [32]. Since safingol is a broad-spectrum inhibitor of PKCs we are unable to precisely identify the steps or isoforms involved, however the similarity between the responses of the cells to PACAP and safingol suggest that further investigation of this pathway may yield an explanation as to why PACAP has immune stimulatory properties in teleosts.

The observation that the VPAC1 inhibitor PG97 also enhanced LPS-induced il-1β expression is also intriguing. There are several differences in VPAC versus PAC1 signaling which may differentially affect their impact on immune function. While both PAC1 and the VPAC1 receptors activate PLC there are differences in the class of G-protein subunits involved [33]. Additionally, PAC1 but not the VPAC receptors can induce activation of the PI3k/Akt signalling pathway [34]. If both or either of these factors are relevant, then biased activation of the PAC1 receptor - either through inhibition of tonic VPAC1 signaling or exogenous administration of PACAP - may explain the increase in il-1β expression seen in these experiments. Alternatively, it may be that the PACAP dosing regime we have selected exhausts elements of the signaling pathway, which in turn leads to effects similar to those seen with inhibitors. This explanation would also be consistent with the changes seen in il-1β expression associated with safingol treatment. However, the differences between il-10 expression changes between PACAP and safingol, along with the fact that we failed to observe any contrary effect at any dose of PACAP administration makes this explanation seem unlikely.

## *Conclusions*

PACAP has attracted some attention as a potential antimicrobial and immune stimulating agent for use in aquaculture. The apparent difference in how PACAP affects the innate immune response in mammals versus teleosts has however remained unexplained despite the increase in research devoted to its use as a therapeutic. This difference may be because PACAP engages receptors differently in fish and mammals, but it may also be that it induces different downstream signalling pathways in these two groups of animals [34]. Previous work has shown efficacy of PACAP when delivered by immersion [12,15] and unpublished work from our group suggest that PACAP could be a useful therapeutant when delivered orally [Fajei et al. in preparation]. Unpublished work also shows that PACAP is not toxic to fish cells are the low concentrations it would be used at commercially, with no toxicity to cell lines below 25, 000 uM [Rivera et al. In preparation]. This work serves as an initial investigation into some of the mechanisms underlying PACAP's effects on teleost immunity. Our results suggest that PACAP is unlikely to be increasing il-1β expression through increased cAMP accumulation. We also found that the PLC inhibitor safingol as well as the VPAC1 inhibitor PG-97-269 had "PACAP-like" effects on il-1β expression. We speculate that, based on the evidence presented here, the effects are a result of preferential activation of the PAC1 receptor and its effects on PLC and /or PI3k/Akt signalling, however more research must be done to further

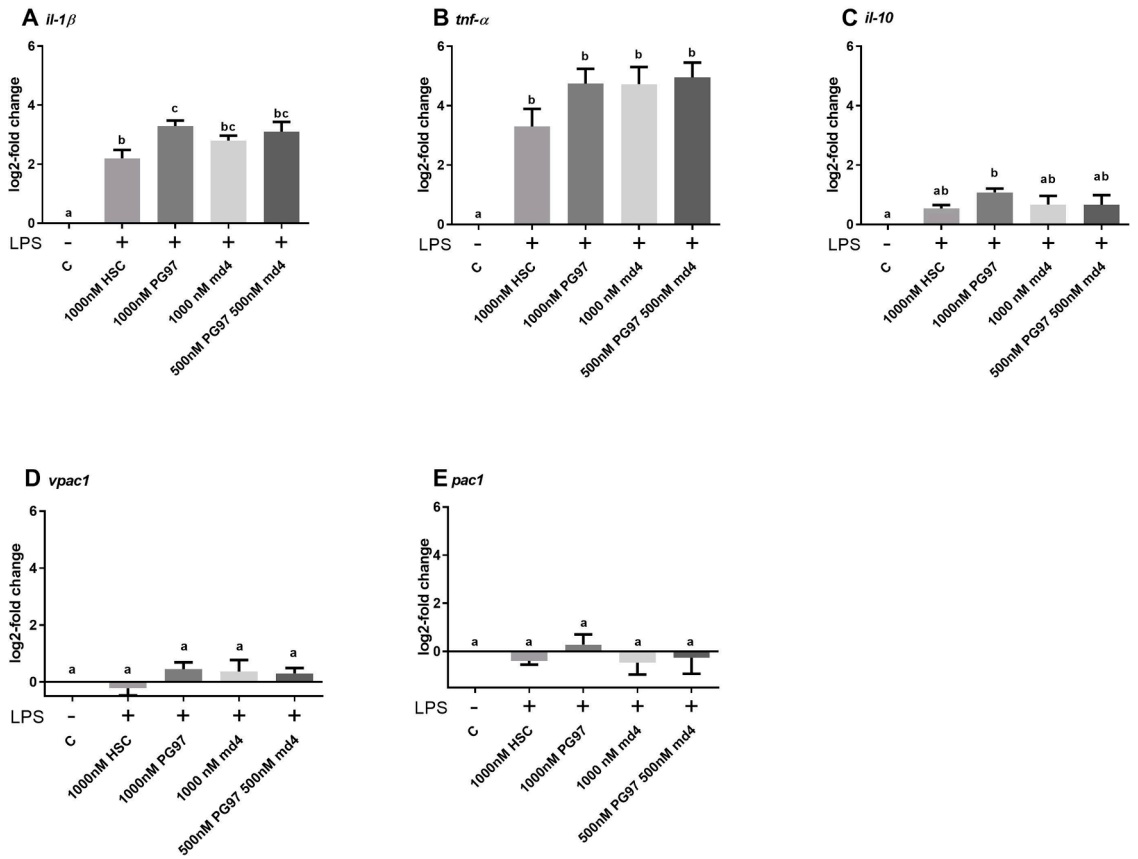


**Fig. 5.** Effect of PAC1 and VPAC1 inhibitors on LPS-induced il-1β (A), tnf-α (B), il-10 (C), vpac1 (D) and pac1 (E) expression in SHK-1 cells. Cells were treated cells were treated with 1000nM of the VPAC1 inhbitor PG97-269 (PG97), with 1000nM of the PAC1 inhbitor max.d.4 (md4), 500nM of both inhibitors or 1000nM of HSC peptide (HSC) 24h and immediately prior to a 4h stimulation with 15 μg/ml LPS. Differences in levels of il-1β, tnf-α and il-10 transcripts as determined by qPCR are presented on the graphs. Data in panels are derived from four independent experiments presented as mean log2 fold change values with standard error of the mean indicated by vertical bars. Lowercase letters denote significant differences at $p < 0.05$.

investigate this supposition. If PACAP is to be used to replace antibiotics as a treatment for the large economic losses due to disease in the aquaculture industry, its mechanism of action needs to be defined.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

## Acknowledgements

This work was carried out with financial support from the UK Government – Department of Health and Social Care (DHSC), Global AMR Innovation Fund (AMRIF) and the International Development Research Centre, Ottawa, Canada. The authors would like to thank Steven Lord for providing the SHK-1 cell line, Lowia Al-Hussinee for his assistance with cell culture work, Aaron Frenette for designing the HSC peptide as well as Manu Soto Davila and Juan Pablo Pontigo for their assistance in primer design.

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