Induction of nitric oxide and respiratory burst response in activated
goldfish macrophages requires potassium channel activity

James L. Stafford\textsuperscript{a}, Fernando Galvez\textsuperscript{a}, Gregory G. Goss\textsuperscript{a}, Miodrag Belosevic\textsuperscript{a,b,*}

\textsuperscript{a}Department of Biological Sciences, University of Alberta, CW-405 Biological Sciences Building, Edmonton, Alta., Canada T6G 2E9
\textsuperscript{b}Medical Microbiology and Immunology, University of Alberta, Edmonton, Alta., Canada T6G 2E9

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Abstract

Potassium channel activity is important for modulating mammalian macrophage antimicrobial functions. The involvement of potassium channels in mediation of immune cell function in lower vertebrates, such as teleost, has not been explored. Since relatively little is known about the types of potassium channels present in fish macrophages, pharmacological blockers with broad ranges of activity were tested: 4-aminopyridine (4-AP), quinine, and tetraethylammonium chloride (TEA). The potassium channel blockers inhibited reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) production by goldfish macrophages activated with bacterial lipopolysaccharide (LPS) and/or macrophage activating factor (MAF)-containing supernatants. Quinine was the most potent inhibitor with an IC\textsubscript{50} of 50 \textmu M, while the other blockers, 4-AP and TEA, had IC\textsubscript{50} of 1.2 and 0.6 mM, respectively. A reversible depolarization of the goldfish macrophage plasma membrane potential (Vm) was observed following treatments with potassium channel blockers, and was related to transcriptional changes in the inducible nitric oxide synthase gene (iNOS). Down-regulation of antimicrobial activities and depolarization of the goldfish macrophage plasma membrane were not a consequence of reduced cell number or viability, suggesting that potassium channels are required for generation of appropriate goldfish macrophage antimicrobial functions. © 2002 Published by Elsevier Science Ltd.

Keywords: Macrophages; Potassium channel; Membrane potential (Vm); Bis-oxyxonol respiratory burst; Nitric oxide; iNOS; Fish

1. Introduction

Teleost macrophages become activated following stimulation with bacterial products (i.e. lipopolysaccharide) and/or soluble host-derived factors [1–3]. We have recently demonstrated that mitogen-stimulated goldfish kidney leukocytes secrete several factors with immunomodulatory properties [4]. These factors were shown to induce the production of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) by activated goldfish macrophages. In addition, a macrophage-deactivating factor (MDF) that inhibited NO production was also characterized and may be the teleost equivalent of mammalian transforming growth factor beta (TGF-\textbeta) [5]. Cytokine-like activities resembling mammalian interferon-\textalpha (IFN-\textalpha), interleukin-1 (IL-1), and IL-2 have also been reported in fish model systems [6–14] and the recombinant mammalian cytokines, TGF-\beta and TNF-\textalpha, appear to functionally cross-react with teleost macrophages [15,16]. Several fish cytokines have also been cloned and sequenced.
including IL-1β, TNF-α, and TGF-β [17–25]. These findings suggest that modulation of fish macrophage antimicrobial function may be regulated through the concerted actions of multiple host-derived factors similar to various cytokines identified in mammals.

Recent work has shown that in addition to pathogen and/or host-derived factors, macrophage antimicrobial functions are modulated by potassium channel activity. Macrophages possess a number of ion channels that may be important for their cytotoxic functions [26–29]. We have recently reported that treatment of activated mammalian macrophages with potassium channel blockers significantly alters their ability to become activated (i.e. inhibition of NO production) [30]. Others have demonstrated the importance of potassium channel activity for mediating immune cell function. For instance, potassium channels were shown to mediate NADPH oxidase activity by human monocyte-derived macrophages [31]. Treatment of human alveolar macrophages with the potassium channel blocker quinine, prevented LPS-induced TNF-α production and treatment of cultured rat microglia with 4-aminopyridine (4-AP) inhibited their activation and subsequent release of IL-1β [32,33]. Activation of T cells and the cytotoxicity of NK cells are also severely impaired following treatments with drugs that block potassium channels [34,35].

To date there are no reports on the role of potassium channels in teleost immune cell function. In this manuscript, we demonstrate for the first time that the antimicrobial functions of goldfish macrophages are impaired following pharmacological modulation of potassium channel activity. The activation pathways induced by bacterial products (i.e. LPS) or host-derived factors (i.e. macrophage activating factor (MAF)) were both susceptible to the effects of these blockers. Treatment of goldfish macrophages with the potassium channel blockers, 4-AP, quinine, and tetraethylammonium chloride (TEA) caused significant reductions in the production of RNI, which was related to the early changes in membrane potential (Vm) and altered transcription of inducible nitric oxide synthase gene (iNOS) gene. Pharmacological blocking of potassium channels also inhibited the respiratory burst response of goldfish macrophages.

2. Materials and methods

2.1. Fish

Goldfish (Carassius auratus) were purchased from either Ozark Fisheries (Southland, MI) or Grassy Forks Fisheries (Martinsville, IN) and maintained at the Aquatic Facility of the Department of Biological Sciences, University of Alberta. The fish were maintained at 20 °C in a flow-through water system on a simulated natural photoperiod (Edmonton, Alberta). Fish were fed to satiation daily with trout pellets, and acclimated to this environment for at least three weeks prior to use in experiments.

2.2. Culture medium

The culture medium used in all experiments has been described by Neumann et al. [36]. Complete medium contained 5% goldfish or carp serum and 10% fetal calf serum (FCS; Hyclone).

2.3. Generation of in vitro derived goldfish kidney macrophages (IVDKM), and production of mitogen-stimulated goldfish kidney leukocyte conditioned supernatants

The generation of IVDKM was described previously [3]. Briefly, fish were anesthetized with MS222 (Syndel), killed by cervical dislocation, and the kidneys aseptically removed and placed in a Petri dish containing incomplete medium. Kidneys were gently passed through sterile mesh screens and the screens were rinsed with 12.5 ml of homogenization buffer/kidney (incomplete medium containing 50 μg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 U/ml heparin). The cell suspension was layered on 51% Percoll (Pharmacia) and centrifuged at 400g for 25 min. Cells at the medium-51% Percoll interface were removed and washed twice in serum-free medium and centrifuged at 200g for 10 min. Viable leukocytes were enumerated using a haemacytometer after staining with trypan blue (Gibco). Isolated leukocytes were used for generation of IVDKM or the production of mitogen-stimulated leukocyte supernatants as described later.

Generation of IVDKM was performed by seeding kidney leukocyte (15–20 × 10⁶) in 20 ml of complete medium supplemented with 25% cell conditioned
medium [3,36]. Cells were incubated at 20 °C and supplemented after 5 days with fresh complete medium. Cultures, 8–12 days old, were harvested, enumerated, and used as a source of macrophages for bioassays.

Mitogen-stimulated goldfish kidney leukocyte supernatants (MAF) were generated using protocols described previously [3,4]. Briefly, isolated kidney leukocytes were pooled, enumerated, and seeded in 75 cm² tissue culture flasks at a concentration of 4 × 10⁶ cells/ml. Kidney leukocytes were incubated 14–16 h in medium containing 2.5% goldfish or carp serum and 10% FCS at 20 °C and following the incubation, the mixed leukocyte cultures were stimulated with 10 μg/ml Concanavalin A (Con A, Boehringer Mannheim), 10 ng/ml phorbol myristate acetate (PMA, Sigma), and 100 ng/ml calcium ionophore A23187 (Sigma). Cultures were incubated in the presence of mitogens for 6 h, after which the mitogens and serum were removed by washing the cells with three exchanges of 25 ml Hanks balanced salt solution (HBSS). The remaining cells were given fresh serum-free medium and incubated for 72 h at 20 °C. After 72 h, the remaining supernatants from all flasks were pooled, filter sterilized, and stored at −20 °C prior to being used in bioassays.

2.4. Effect of potassium channel blocker treatments on nitric oxide and respiratory burst response of goldfish macrophages

Goldfish macrophages (IVDKM) (5 × 10⁴/well) were seeded into 96-well half-area tissue culture plates (Costar) in 50 μl of complete medium. Twenty-five microlitres of different potassium channel blockers were added to wells in triplicate: 4-AP, or quinine, or TEA at a final concentration of 2.5 mM, 200 μM, and 10 mM, respectively. Macrophages were subsequently stimulated with 25 μl of LPS (10 μg/ml final), MAF (1:4), or MAF (1:4) and LPS (1 μg/ml final) and incubated for 72 h at 20 °C prior to determination of nitrite production. A control group was incubated with medium instead of potassium channel blockers and treated with LPS and/or MAF as described earlier. For some experiments, time-course of nitrite production was determined in the absence or presence of potassium channel blockers. Cells were seeded, treated with the blockers, and stimulated with 10 μg/ml LPS as described earlier. Nitrite production was determined using the Griess reaction after 12, 24, 48, and 72 h incubation at 20 °C.

Dose-dependent inhibition of nitrite production by potassium channel blockers was determined by seeding goldfish macrophages (5 × 10⁴ per well) into half-area 96-well culture plates (Costar) and treating triplicate cultures with a range of doses of 4-AP (0–2.5 mM), quinine (0–200 μM) or TEA (0–10 mM). Cells were simultaneously stimulated with LPS (10 μg/ml) and incubated for 72 h at 20 °C prior to determination of nitrite production using the Griess reaction.

The production of ROI in the presence of potassium channel blockers was also determined by seeding goldfish macrophages (5 × 10⁴ per well) into 96-well half-area culture plates (Costar) and treating quadruplicate cultures with 4-AP (2.5 mM) followed by priming with MAF (1:4) and LPS (1 μg/ml). Cells were incubated for 6 and 24 h at 20 °C prior to determination of ROI production using the NBT reduction assay. Cells were also simultaneously examined for relative changes in Vm as described later.

2.5. Effect of potassium channel blocker treatment on relative membrane potential (Vm) changes of goldfish macrophages

To determine the effects of potassium channel blockers on relative changes of macrophage plasma membrane Vm, the fluorescent anionic dye bis-oxonol (Molecular Probes) was used. This negatively charged dye is excluded by the mitochondria and prevents complications of signals derived from mitochondrial origin [37]. Cells were seeded in triplicate (2.5 × 10⁵ per tube) into 5 ml polypropylene tubes (Falcon) in 250 μl of medium, and treated with 125 μl of medium (control) or 125 μl of 4-AP (2.5 mM final), quinine (200 μM final), or TEA (10 mM final). Final volume in all tubes was 500 μl with a final concentration of 2% FCS. After 30 min, 2.5, and 5.5 h, cells were loaded with 5 μM of bis-oxonol and incubated for a further 25 min at 20 °C. Cells were then washed two times with 3 ml medium (control) or with medium supplemented with potassium channel blockers to remove residual bis-oxonol. Cells were re-suspended in a final volume of 250 and 50 μl (5 × 10⁴ cells) were plated in triplicate into wells of half-area 96-well
culture plates (Costar). The relative fluorescence intensity per 5 × 10⁴ goldfish macrophages was determined (excitation 544 ± 15 nm; emission 590 ± 14 nm; 60 ms integration time) using a fluorescence microplate spectrophotometer (Molecular Dynamics fmax).

2.6. Reversibility of altered goldfish macrophage Vm by removal of potassium channel blockers

To determine if the potassium channel blockers used in this study were directly responsible for alterations of goldfish Vm, potassium channel blocker wash-out experiments were performed. Briefly, cells were seeded and incubated with 2.5 mM 4-AP or 200 μM quinine for 3 h. Prior to the addition of 5 μM bis-oxonol, the blockers were removed from the wash-out group by washing the cells two times with 3 ml medium. After washing, the cells were loaded with bis-oxonol as indicated earlier, incubated 25 min at 20 °C, washed two times with 3 ml medium to remove bis-oxonol and resuspended in 250 μl of medium. Fluorescence (FL-2) was determined using flow cytometry (FACS Calibur flow cytometer; Becton Dickinson). Flow cytometry was performed by collecting 10,000 cells during analysis with the following settings; forward side scatter-photodiode set to E-1, AmpGain set to 9.33; and side scatter-photomultiplier voltage set to 455 V, AmpGain set to 1.00.

2.7. iNOS expression by stimulated goldfish macrophages following treatments with potassium channel blockers

The effect of potassium channel blocker treatments on goldfish macrophage iNOS was determined using Northern blot analysis and non-isotopic labeling and detection kits (Ambion) described later.

2.7.1. Reverse transcriptase-mediated polymerase chain reaction (RT-PCR)

RT-PCR was used to generate PCR products for non-isotopic labeling. Briefly, goldfish macrophages were incubated in control medium or stimulated with LPS (10 μg/ml) and incubated for 6 h at 20 °C prior to total RNA extraction using TriZol (Gibco), according to the manufacturers instructions. Total RNA (2.5 μg) was used for first-strand cDNA synthesis using RT-PCR (Stratagene) by incubating with 1.5 μl of oligo (dT) primer (100 ng/μl) at 65 °C for 5 min. The reaction was cooled at room temperature for 10 min to allow primers to anneal to the RNA after which the following components were added to the reaction in order; 2.5 μl of 10× first strand buffer, 0.5 μl of RNase Block Ribonuclease Inhibitor (40 U/μl), 1.0 μl of 100 mM dNTPs, and 1.0 μl of MMLV-RT (50 U/μl). The reagents were gently mixed and incubated for 1 h at 37 °C. The reaction was terminated by heating at 90 °C for 5 min and the cDNA stored at −20 °C prior to use in PCR reactions.

The PCR reactions used to amplify iNOS and β-actin PCR products were performed using the following primer pairs ordered from Gibco:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>Forward 5’ GGAGGTACGTCGCGAGAAGCT 3’</td>
</tr>
<tr>
<td>iNOS</td>
<td>Reverse 5’ CCAGCCTGCAAACCTATCATCCA 3’</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward 5’ CGAGGCTGCGTGTCGCCCCCTGAG 3’</td>
</tr>
<tr>
<td>β-actin</td>
<td>Reverse 5’ CGGCCGTGGTGTGAAGCTGTA 3’</td>
</tr>
</tbody>
</table>

The iNOS primer pairs were designed to amplify a 600 bp product and the β-actin primer pairs were designed to amplify a 350 bp product. To amplify iNOS and β-actin products, PCR was performed by combining the following reagents (Roche) in order into a master mix such that each reaction contained the following: 0.5 μl of 10× dNTPs (10 mM), 2.5 μl of 10× PCR buffer (with 1.5 mM MgCl₂), 0.25 μl of Taq DNA polymerase (5 U/μl), and 20.25 μl of sterile H₂O. The master mix was gently mixed and spun briefly, then 23.5 μl was added to each tube followed by 0.5 μl of either iNOS Forward and Reverse or β-actin Forward and Reverse primers (70 pmoles final concentration). Finally, 0.5 μl of the cDNA template was added and samples placed immediately in a thermocycler and amplification was performed using the following cycling parameters: (1) 94 °C, 3 min; (2) 94 °C, 35 s; (3) 62 °C, 35 s; (4) 72 °C, 35 s; 30 cycles of step (2)–(4); (5) 72 °C, 10 min. Immediately following PCR amplification, 8 μl of the PCR sample was run on a 1.6% agarose/TAE gel for confirmation of the size of PCR products. The remainder of the PCR products was run through GFX™ PCR DNA Purification Columns (Pharmacia) to remove unincorporated nucleotides and excess salts. The purified PCR products were resuspended to a final concentration of 50 ng/μl and stored at −20 °C prior to non-isotopic labeling.
2.7.2. Production of iNOS and β-actin probes for northern blot analysis

The purified PCR products were labeled using the BrightStar™ Psoralen-Biotin Non-isotopic Labeling Kit (Ambion) according to the manufacturers protocols. Briefly, 10 µl (500 ng) of PCR products were heat denatured for 10 min at 100 °C followed by immediate quick-chill by placing the samples in an ice-water bath. Freshly denatured DNA samples were labeled by the addition of 1 µl of psoralen-biotin reagent in the well of a microtiter plate (Costar). The plate was placed on ice and irradiated with a UV lamp set to 365 nm for 45 min. The sample was then diluted up to 100 µl by the addition of 89 µl of 1 x TE buffer and transferred to a clean microcentrifuge tube. Non-cross linked psoralen-biotin reagent was removed by two rounds of butanol extractions and the non-isotopic labeled probes were aliquoted into 20 µl samples and stored at −80 °C. The final concentration of the probes was approximately 5 ng/µl.

2.7.3. Northern blot analysis of goldfish iNOS expression

Goldfish macrophages were seeded into 5 ml polypropylene tubes (Falcon) (2.5 x 10⁶ per tube) in 250 µl of complete medium. Cells were stimulated with 125 µl LPS (10 µg/ml final concentration) and treated with 125 µl medium (control) or 125 µl 4-AP (2.5 mM final concentration) for a final volume of 500 µl. Total RNA was isolated from the cells after 0, 2, 4, 6, 12 and 24 h incubation at 20 °C and stored at −80 °C.

Northern blot analysis was performed using the NorthernMax™ kit (Ambion) according to the manufacturers protocols. Briefly, RNA samples (10 µg/lane) were diluted with 3 volumes of formaldehyde load dye (Ambion) and incubated at 65 °C for 15 min to denature RNA secondary structure. Samples were then loaded onto a 0.6 mm thick, 1% formaldehyde agarose gel and electrophoresed for 1.5 h at 125 V. Following electrophoresis, RNA was transferred to Ambion’s BrightStar-Plus™ positively charged nylon membranes using a downward transfer assembly. The RNA was cross linked in a UV Stratalinker 2400 (Stratagene) at 120,000 µJ, and the membrane pre-hybridized for 1 h at 42 °C using ULTRAhyb™ hybridization solution provided by Ambion. Non-isotopic probes (e.g. iNOS and β-actin) were heat denatured by diluting in 50 µl 10 mM EDTA and incubating at 90 °C for 10 min and added directly to the appropriate pre-hybridized membrane. Probe and membrane were allowed to hybridize overnight (16 h) at 42 °C with gentle agitation. Hybridization solution was removed and the membranes were washed with two 5 min washes with 2 x SSC/0.1% SDS (low stringency) at room temperature followed by two 15 min washes with 0.1 x SSC/0.1% SDS (high stringency) at 42 °C. Membranes were briefly blotted to remove excess wash solution and RNA expression detected using the BrightStar™ BioDetect™ non-isotopic detection kit (Ambion) following the manufacturer’s protocols. The non-isotopic labeled probes were visualized using a chemiluminescent detection system followed by exposure of the membrane to film (Kodak) at room temperature. Specific exposure times of the membranes are indicated in Section 3.

2.8. Effects of potassium channel blocker treatment on the viability of goldfish macrophages

Goldfish macrophages were seeded in triplicate into 6 ml tubes at a cell density of 3.0 x 10⁶ cells in 250 µl. Cells were then treated with the potassium channel blockers 4-AP, TEA and quinine at final concentrations of 2.5, 10 mM, and 200 µM, respectively. Macrophages treated with medium alone were used as the control group. Cells were incubated for 24, 48, and 72 h at 20 °C, and at each time point cell number and viability of triplicate cultures was assessed by trypan blue staining and enumerating cells using a haemocytometer.

2.9. Respiratory burst assay

The O₂ production of goldfish macrophages was measured using the nitroblue tetrazolium (NBT) reduction assay [38]. IVDKM were seeded into half-area 96-well culture plates (Costar), and treated in triplicate with either 0 mM 4-AP (control) or 2.5 mM 4-AP and stimulated with MAF (1:4) and LPS (1 µg/ml). Cultures were then incubated for 6 or 24 h prior to determination of NBT reduction. Briefly, NBT was dissolved in dimethyl sulphoxide (DMSO, 20%, v/v) and pre-warmed (50 °C) Dulbecco’s PBS (Gibco) was added to completely dissolve NBT to a final concentration of 2 mg/ml. This NBT solution was heated for a further 10 min at 50 °C and filter
sterilized. PMA was added to the filtered NBT solution at a final triggering concentration of 50 ng/ml. Fifty microlitres of the NBT/PMA solution was added to the macrophage cultures and the plates centrifuged at 200g for 1 min to pellet cells. Cultures were incubated for 25 min at room temperature, followed by removal of the supernatant. Macrophages were fixed by adding 200 µl of 70% methanol and incubating for 1 min. Unreduced NBT was removed by washing cells several times with 70% methanol. Reduced NBT was dissolved by adding 60 µl of 2 M KOH to individual wells followed by vigorous pipetting. Seventy microlitres of DMSO was added to all wells and the optical densities read at 630 nm on a microtitre plate reader (Biotek). Sixty microlitres of 2 M KOH and 70 µl of DMSO were added to wells without cells and used as blanks.

2.10. Nitric oxide assay

Goldfish macrophages were seeded (5 × 10⁴ cells/ml) into 96-well half-area culture plates (Costar) and treated as indicated earlier. NO produced by the stimulated macrophages was analyzed by sampling the supernatants after 72 h incubation for the presence of nitrite, using the Griess reaction [39]. Briefly, 75 µl of supernatants were removed from individual wells and placed in a separate 96-well microtitre plate. One hundred microlitres of 1% sulphamamide in 2.5% phosphoric acid was added to each sample followed by 100 µl of 0.1% N-naphthyl-ethylenediamine in 2.5% phosphoric acid. The optical density of each well was determined using an automated plate reader (Biotek) at 540 nm. The approximate concentration of nitrite in the samples was determined from a standard curve generated using known concentrations of sodium nitrite.

2.11. Statistics

One and two-way analysis of variance using Super-Anova software (Abacus) for the Power Macintosh was used for determining significance between control and experimental groups. Probability level of p < 0.05 was considered significant.

Fig. 1. Dose-response inhibition of NO production by potassium channel blockers. Goldfish macrophages (5 × 10⁴ per well) were treated with varying doses of the potassium channel blockers 4-AP, quinine, and TEA as described in Section 2. Nitrite production was determined in each treatment group after 72 h incubation at 72 °C using the Griess reaction. Each bar represents the mean ± SEM of triplicate cultures and the data are from a representative experiment from three independent experiments performed.
3. Results

3.1. Dose-dependency of NO inhibition induced by potassium channel blockers

The potassium channel inhibitors 4-AP, quinine, and TEA caused a dose-dependent decrease in NO production of goldfish macrophages (Fig. 1). There were significant differences in the potency of goldfish macrophages to the various potassium channel inhibitors. Quinine was the most potent inhibitor of NO production in goldfish macrophages with an IC\textsubscript{50} of only 50 \mu M. In contrast, 4-AP and TEA were relatively weak inhibitors of NO production with IC\textsubscript{50} values of 1.2 mM and 0.6 mM, respectively. At the highest doses tested, 4-AP, quinine, and TEA caused 80, 70, and 50% inhibitions of NO production by goldfish macrophages.

3.2. Potassium channel blockers significantly inhibit NO production by goldfish macrophages stimulated with LPS and/or host-derived factors

Potassium channel blockers significantly inhibited NO production independent of the activation signal. Goldfish macrophages activated with bacterial LPS, MAF, or MAF and LPS had significantly higher levels of NO relative to untreated cells (Fig. 2). Treatment of activated macrophages with potassium channel blockers resulted in significant reductions in NO production (4-AP and quinine; \( p < 0.0001 \)) compared to controls. Cells treated with 10 mM TEA demonstrated significant reductions in NO production only following activation with LPS (10 \mu g/ml) (\( p < 0.0001 \)). Potassium channel blockers alone did not induce nitrite production when added to fish macrophage cultures (Fig. 2, cells alone). These results were not due to decrease in macrophage viability or cell numbers following treatment with the potassium channel blockers (Table 1).

3.3. Time-course production of NO by stimulated goldfish macrophages treated with potassium channel blockers

Goldfish macrophages were stimulated with LPS (10 \mu g/ml) and incubated for 72 h at 20 °C in medium or in the presence of 2.5 mM, 200 \mu M, and 10 mM of 4-AP, quinine, and TEA, respectively. After 12, 24, 48, and 72 h, supernatants were removed from the cultures and examined for NO production using the Griess reaction. As shown in Fig. 3, activated goldfish macrophages produced detectable levels of NO between 24 and 48 h, which increased after longer incubation. In the presence of potassium channel blockers 4-AP and TEA, NO was
Table 1
Effect of potassium channel blockers on viability and total cell number of goldfish macrophages

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>Incubation time (h)</th>
<th>Cell number (× 10^5)</th>
<th>% Viableb</th>
<th>p valuec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24</td>
<td>31 ± 1</td>
<td>&gt; 95</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>26 ± 2</td>
<td>&gt; 95</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>34 ± 1</td>
<td>&gt; 95</td>
<td>–</td>
</tr>
<tr>
<td>2.5 mM 4-AP</td>
<td>24</td>
<td>31 ± 2</td>
<td>&gt; 95</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>31 ± 1</td>
<td>&gt; 95</td>
<td>0.114</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>31 ± 3</td>
<td>&gt; 95</td>
<td>0.365</td>
</tr>
<tr>
<td>10 mM TEA</td>
<td>24</td>
<td>34 ± 1</td>
<td>&gt; 95</td>
<td>0.342</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>30 ± 3</td>
<td>&gt; 95</td>
<td>0.174</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>31 ± 1</td>
<td>&gt; 95</td>
<td>0.378</td>
</tr>
<tr>
<td>200 μM quinine</td>
<td>24</td>
<td>34 ± 2</td>
<td>&gt; 95</td>
<td>0.319</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>31 ± 2</td>
<td>&gt; 95</td>
<td>0.109</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>30 ± 1</td>
<td>&gt; 95</td>
<td>0.141</td>
</tr>
</tbody>
</table>

a Highest doses of potassium channel blockers with maximal inhibitory activity were chosen for viability experiment.
b Trypan blue exclusion was used for determination of cell numbers and macrophage viability using the formula: % Viable cells = [number of live cells − number of dead cells]/number of live cells]100.
c p value when comparing potassium channel blocker treated cells with the control group of cells.

3.4. Relative changes of goldfish macrophage membrane potential (Vm) following treatments with potassium channel blockers

It has been previously shown that the membrane potential (Vm) of mammalian macrophages is significantly reduced but detectable after 48 h (p < 0.05) and also significantly reduced at 72 h (p < 0.0001). Macrophages treated with quinine only produced detectable levels of NO after 72 h which was significantly lower than control values (p < 0.0001).

![Nitrite production graph](image)

Fig. 3. Time-course of NO production by goldfish macrophages in the absence or presence of potassium channel blockers. Goldfish macrophages (5 × 10^7 per well) were treated with 2.5 mM, 200 μM, and 10 mM of 4-AP, quinine, and TEA, respectively. Cells were simultaneously activated with LPS (10 μg/ml) and supernatants removed after 12, 24, 48, and 72 h incubation at 20 °C and analyzed for NO production using the Griess reaction. Each bar represents the mean ± SEM of triplicate cultures and the data are from a representative experiment from three independent experiments performed.
modulated predominantly by the activity of potassium channels on the plasma membrane [27]. As a result, changes in plasma membrane Vm would be representative of alterations in the function of potassium channels. To measure the effects of potassium channel blockers on plasma membrane Vm, we used the fluorescent dye bis-oxonol. Bis-oxonol slowly distributes across biological membranes according to the Vm and binds to hydrophobic components. Since the quantum yield of the dye increases significantly upon binding, the relative fluorescence (RFU) of cells incubated in medium containing the dye increases upon depolarization and decreases with hyperpolarization [37]. In the present study, it was observed that the RFU of untreated goldfish macrophages, loaded with 5 μM bis-oxonol, ranged between 60 ± 20 RFU per 5 x 10^3 cells when using the fluorimeter and demonstrated a wider range of fluorescence intensities when using the flow cytometer (400 ± 150 per 10,000 cells). To compensate for variations in the loading efficiency of bis-oxonol and to allow for comparisons between independent experiments, we normalized the RFU values such that the untreated control group had an RFU value of 1.0. Changes in the RFU of macrophages in medium containing bis-oxonol is an indication of relative changes in plasma membrane Vm, with a value >1.0 representing depolarization and a value <1.0 representing hyperpolarization. Treatment with 4-AP (2.5 mM), quinine (200 μM) or 10 mM TEA each significantly depolarized (REF > 1.0; p < 0.0001) the plasma membrane potential compared to untreated controls (Fig. 4). Vm depolarization by these potassium channel blockers was observed at the earliest time point tested in this study (1 h) and was sustained for at least 24 h (Fig. 4 and Table 2). Compared to 4-AP and quinine, TEA caused relatively smaller changes of macrophage Vm at all time points examined. Activation of goldfish macrophages with MAF and/or LPS did not cause any significant changes in Vm compared with control cells (data not shown).

3.5. Reversibility of plasma membrane depolarization following removal of potassium channel blockers

Treatment of goldfish macrophages with potassium channel blockers caused significant depolarization of the plasma membrane (Fig. 4). To determine if this effect was directly due to treatments with the potassium channel blockers, wash-out experiments were performed. The blockers 4-AP (2.5 mM) and quinine (200 μM) were used since they caused the greatest
changes in Vm (Fig. 4). Shown in Fig. 5 is the effect of a 3 h treatment with 4-AP (2.5 mM) or quinine (200 μM) followed by removal of the drug. Control cells stained with bis-oxonol demonstrated a shift in fluorescence intensity (FL-2) compared to unstained cells (Fig. 5, upper panel). Incubations in the presence of 4-AP or quinine caused a significant \( p < 0.0001 \) increase in FL-2 fluorescence (i.e. depolarization) which was reversed by washing-out the blockers. The fluorescence intensities (FL-2) of control cells, potassium channel blocker-treated, and wash-out groups are summarized (lower panel) which demonstrates the reversibility of Vm changes caused by potassium channel blockers (Fig. 5).

### 3.6. Blocking potassium channels caused reduced respiratory burst activity of goldfish macrophages

Goldfish macrophages treated with 2.5 mM 4-AP and primed with MAF and LPS demonstrated significant reductions in ROI production after 6 and 24 h (Table 2). The reductions in respiratory burst activity after 6 and 24 h were 21.9 and 39.6% (Exp 1), 17.3 and 39.9% (Exp 2), and 20.6 and 26.2% (Exp 3), respectively (Table 2). Relative changes in macrophage Vm were simultaneously examined using bis-oxonol fluorescence. After 6 and 24 h the plasma membrane of macrophages treated with 4-AP were significantly depolarized (i.e. RFU > 1.0; \( p < 0.0001 \) when compared to untreated controls. Potassium channel blocker treated macrophages demonstrated a 2–3 fold increase in bis-oxonol RFU at both time points (6 and 24 h) for all three experiments (Table 2).

### 3.7. Effect of potassium channel blocking on the transcription of iNOS by stimulated goldfish macrophages

Fig. 6A shows a 1.6% EtBr-stained gel of the PCR products produced from β-actin and iNOS primer pairs designed against goldfish sequences. The expected sizes of the fragments for both products matched, based on the regions flanked by forward and reverse primers. Relative changes in the expression of iNOS were also observed since unstimulated cells (lane 3) contained no PCR product, whereas after stimulation (lane 4) a PCR product was detected. Lanes 1 and 2 are β-actin PCR products from unstimulated and stimulated goldfish macrophages, respectively. These PCR products were used for production of non-isotopic probes for Northern blot analysis.
The relative changes in iNOS expression by activated goldfish macrophages treated with potassium channel blockers using Northern blot analysis is shown in Fig. 6A and B. Fig. 6B shows the time-course induction of iNOS after stimulation with LPS (10 μg/ml) in the absence (control; 0 mM 4-AP) or presence of 2.5 mM 4-AP. Detectable levels of iNOS expression were observed after 6 h in the control group, which peaked after 12 h, and declined slightly by 24 h. Cells treated with 2.5 mM 4-AP, which were previously shown to have significantly reduced NO production (Figs. 1–3), appeared to have a relative increased expression of iNOS which was significantly higher after 24 h. These increased levels of iNOS expression were not an artifact of RNA loading as demonstrated by β-actin expression. The other blockers used in this study (quinine and TEA), also appeared to increase iNOS expression after 12 and 24 h as shown in Fig. 6C.
TEA acts by binding to a specific receptor site on the extracellular side of the potassium channel and physically blocks the pore [26,40,41]. Delayed-rectifier type, inward rectifier type, calcium-sensitive type and ATP-sensitive type potassium channels are all affected by TEA. The inhibitor 4-AP is lipid-soluble and membrane permeable, which crosses plasma membranes to reach its site of action [26]. Within macrophages, 4-AP physically blocks the pore of the delayed-rectifier type and voltage-sensitive potassium channels. Quinine is an alkaloid isolated from plants of the Cinchona family and like 4-AP, is a membrane-permeant molecule that blocks potassium channels by passing into the cytoplasm of cells or diffusing from the cell membrane to bind the receptor within the pore and physically block the passage of potassium ions [42–44]. Quinine blocks delayed-rectifier, calcium-sensitive, and ATP-sensitive type potassium channels of macrophages. All of these blockers significantly inhibited goldfish macrophage activation. The wide variety of potassium channels that are likely present on goldfish macrophages suggests that each type contributes a small amount to activation processes. Interestingly, TEA failed to significantly inhibit NO production by goldfish macrophages stimulated with MAF alone or in combination with LPS, but significantly inhibited NO production by macrophages stimulated with LPS alone. Conversely, quinine and 4-AP inhibited NO production by cells stimulated with LPS and/or MAF. This suggests that the activation pathway(s) triggered by LPS and MAF may be different and appeared to be mediated by different types of potassium channels.

The major role of potassium channels within cells is the establishment and maintenance of Vm [27,45]. Alterations in the activity of potassium channels results in changes in Vm (i.e. depolarization or hyperpolarization), which could have significant effects on proteins and enzymes embedded in the plasma membrane [46]. Modulation of the activities of functional enzymes found in the plasma membrane can significantly affect immune cell functions. For instance, potassium channel activity was shown to mediate the activity of the membrane bound NADPH oxidase of human macrophages [31]. Activation of this enzyme, which catalyzes superoxide formation and production of ROI, was dependent on Vm that was directly modulated by the opening of calcium-dependent potassium

4. Discussion

In this study, we demonstrated for the first time that fish macrophage antimicrobial functions are affected by potassium channel activity. Compared to the information regarding mammalian macrophage potassium channels, relatively little is know about the types of potassium channels found on fish macrophages. Therefore, pharmacological blockers that affect numerous types of potassium channels through a variety of different mechanisms were used in this study.
channels. In addition, TEA inhibited the PMA-stimulated respiratory burst in microglia and charybdotoxin dramatically inhibited the respiratory burst by cultured macrophages stimulated by opsonized zymosan [31,47]. These data further support the hypothesis that potassium channels regulate NADPH oxidase activity but little is known about the mechanism of enzyme inhibition. A rise in intracellular Ca\(^{2+}\) is important for the full activation of the respiratory burst and Ca\(^{2+}\) channels have been implicated in this response [48–50]. Macrophages have Ca\(^{2+}\) channels that are activated by depletion of intracellular stores (Ca\(^{2+}\) release-activated Ca\(^{2+}\) channels) rather than depolarization-dependent gating [51]. In this case, Ca\(^{2+}\) entry is increased by hyperpolarization and the role of potassium channels would be to counteract the depolarizing Ca\(^{2+}\) current and maintain the driving force for Ca\(^{2+}\) influx required for NADPH oxidase activity [52]. Therefore, blocking potassium channels results in alteration of the activity of other ion channels or exchange pumps (i.e. Ca\(^{2+}\) channels). How potassium channel blocking causes reduced NADPH oxidase activity in fish macrophages is unknown but may be similar to mechanism described for mammalian macrophages.

In the present study, we also showed that potassium channel blockers can inhibit production of NO by fish macrophages indicating that these channels are important for fish macrophage activation. We hypothesized that reduced NO production would correlate with reduced expression of iNOS mRNA. Following treatments with potassium channel blockers, the expression of goldfish macrophage iNOS levels were surprisingly increased. These results suggest that the significant reduction of NO production, following blockage of potassium channels were not due to suppressed iNOS. The observed increased levels of iNOS transcription by potassium channel blockers and subsequent reduction in NO production may be explained by postulating an absence of a negative regulatory feedback mechanism. Regulation of iNOS enzyme synthesis and RNA transcription is tightly controlled (reviewed in Ref. [53]). Recently, it has been demonstrated that end-products produced by iNOS enzyme activity (i.e. NO and peroxynitrite) can inhibit iNOS mRNA expression, iNOS enzyme synthesis and enzyme activity [54–56]. Both NO generated intracellularly following iNOS induction or released from NO donors limited the transcription of the iNOS gene. Interestingly, removal of the negative feedback signal (i.e. NO) by inhibition of iNOS enzyme activity, resulted in an increase of iNOS mRNA expression [54]. Thus, the observed increase of goldfish iNOS mRNA expression after treatment with potassium channel blockers may be due to the impairment of the negative feedback signal, due to significantly reduced NO levels.

Our results demonstrate that goldfish macrophage NO production and respiratory burst activity are inhibited by potassium channel blockers. The mechanism(s) responsible for these inhibitory responses are unknown. However, the direct effect of these blockers on potassium channel activity caused subsequent depolarization of the plasma membrane, which may lead to impairment of intracellular signaling steps required for macrophage activation. Recently, there has been a direct link established between LPS-activation of macrophages, potassium channel activity, and transmembrane signaling [57]. This report demonstrated that activation of potassium channels was an early step in the transmembrane signal transduction in macrophages, which could be inhibited by potassium channel blockers. Therefore, modulation of potassium channels alters LPS-induced transmembrane signaling required for gene transcription (i.e. TNF-α). This recent report may explain why treatment of human alveolar macrophages with quinine prevented LPS-induced TNF-α release and the findings of another group who showed that the release of TNF-α by LPS-stimulated mouse macrophages was dependent on Vm [32,58]. A similar cascade of events may also occur in fish macrophages. Goldfish macrophages respond to LPS stimulation by up-regulating the expression of a variety of genes (i.e. iNOS, TNF-α, and IL-1). Reduced production of cytokines and antimicrobial factors (i.e. NO and ROI) by fish macrophages, following perturbation of potassium channels would significantly impair antimicrobial functions of macrophages. Further investigations are required to fully understand the role of potassium channels in teleost immune cell function and identify factors that may alter potassium channel activity.

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