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Ca^{2+} SIGNALS DURING EARLY LYMPHOCYTE ACTIVATION IN CARP *CYPRINUS CARPIO* L.

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Summary

To measure cellular responses and the involvement of increased cytosolic Ca^{2+} levels ($[Ca^{2+}]_i$), peripheral blood leukocytes (PBL) of carp were loaded with the fluorescent intracellular Ca^{2+} indicators Fluo-3 and Fura-2. Responses of lymphocytes to T-cell mitogen (phytohaemagglutinin, PHA), to B-cell mitogen (lipopolysaccharide, LPS) and to immunoglobulin (Ig) cross-linking with a monoclonal antibody to carp Ig were measured using flow cytometry. Both T-cell stimulation by PHA and B-cell stimulation by membrane Ig cross-linking evoked a rapid elevation of $[Ca^{2+}]_i$. B-cell stimulation by LPS was not linked to an increase in $[Ca^{2+}]_i$. As judged by the percentage of reacting cells, it was concluded that all Ig-positive lymphocytes reacted to Ig cross-linking by elevating $[Ca^{2+}]_i$. At the single-cell level, the reactions of Fura-2-loaded cells were followed every 6 s using digital imaging microscopy. Both cells displaying spontaneous $[Ca^{2+}]_i$ oscillations and non-oscillating cells responded to stimulation with an increase in $[Ca^{2+}]_i$, sometimes, in already oscillating cells, accompanied by an increase in frequency and/or amplitude of the oscillations. These results show that intracellular Ca^{2+} responses of PBL upon activation resemble those in mammals and form a powerful tool for studies into cell-specific regulation.

Key words: fish, carp, lymphocytes, intracellular Ca^{2+}, phytohaemagglutinin, lipopolysaccharide, B-cell cross-linking, Fluo-3, Fura-2, flow cytometry, digital imaging microscopy, *Cyprinus carpio*.

Introduction

In mammals, lymphocyte activation or inhibition results from a multitude of immune and non-immune signals. Positive and negative signals evoke a delicate interplay of signal transduction pathways in the target lymphocytes which will eventually determine cellular responses, e.g. cell proliferation, cytokine secretion or apoptosis. Cytosolic Ca^{2+} signals play a pivotal role in signal transduction and can be generated by Ca^{2+} influx and/or by release of Ca^{2+} from the endoplasmic reticulum (Berridge, 1993; Clapham and Sneyd, 1995). Both initiation of proliferation and apoptosis in response to cross-linking of membrane Ig have been demonstrated to be paralleled by an increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in mammalian B-cells (Cambier, 1992). In mammalian T-cells, neurotransmitters, hormones and growth factors act through G-protein- or tyrosine-kinase-linked receptors to promote the formation of the second messenger inositol 1,4,5,-trisphosphate (InsP_3), which then diffuses into the cytosol to release Ca^{2+} from the endoplasmic reticulum by activating InsP_3-operated Ca^{2+} channels (Cambier, 1992; Weiss, 1993). The Ca^{2+} signal is rapidly terminated by the action of Ca^{2+} pumps, which allows the realisation of brief Ca^{2+} spikes (oscillations) and waves (Clapham, 1995). However, the physiological relevance of these oscillatory changes in $[Ca^{2+}]_i$ is still poorly understood.

In fish, research is at the brink of discriminating functionally different lymphocyte (sub)populations. Fish lymphocytes can now be subdivided into surface immunoglobulin positive (sIg^+) and negative (sIg^-) cells. The sIg^+ population is specifically sensitive to the B-cell mitogen lipopolysaccharide (LPS), whereas consensus exists that the sIg^- population is specifically sensitive to the T-cell mitogen phytohaemagglutinin (PHA) (Koumans-van Diepen *et al.* 1994). For carp, monoclonal antibodies are available for B-cells (anti Ig) (Secombes *et al.* 1983), monocytes (Weyts *et al.* 1997*a*) and thrombocytes (Rombout *et al.* 1996). Antibodies for discrimination of O-cells and cells responsible for helper (CD4-like) and suppresser (CD8-like) function are as yet lacking.

Most of the factors involved in the regulation of the different fish lymphocyte subtypes are unknown. For instance, fish interleukins still await identification. In this context, the recent finding that membrane immunoglobulin cross-linking leads to activation of the InsP_3 pathway in catfish Ig^+ cells (van Ginkel

et al. 1994) is of importance, since it shows that, in principle, measurement of $[Ca^{2+}]_i$ can provide a powerful means to define and identify the factors involved in lymphocyte activation. It is to be expected that this will greatly enhance our concept of immune homeostasis and its regulation in fish.

We report here on cytosolic Ca^{2+} signals following *in vitro* stimulation of carp lymphocytes. Using flow cytometry of cells loaded with Fluo-3 and digital imaging microscopy of cells loaded with Fura-2 to monitor the dynamics of cytosolic Ca^{2+} signalling in suspensions of cells and individual cells, respectively, activation of fish Ig^- lymphocytes by PHA and activation of Ig^+ lymphocytes by LPS and Ig cross-linking was studied.

Materials and methods

Animals

Common carp, *Cyprinus carpio* L., were obtained from laboratory stock ('De Haar Vissen', Agricultural University, Wageningen, The Netherlands). Fish were the offspring of a hybrid cross (WAUR3×WAUR8) between two parents, the female of Polish origin (R3 strain) and the male of Hungarian origin (R8 strain). Both strains represented the seventh generation. Animals were kept at 23 °C in recirculating ultraviolet-sterilized water and fed daily with pelleted dry carp food (Provimi, Rotterdam, the Netherlands). Adult (11–16 months old) fish of both sexes, weighing approximately 220 g, were used. Animals were anaesthetised with 0.2 g l^{-1} tricaine methane sulphonate (Crescent Research Chemicals, Phoenix, AZ, USA), adjusted to pH 7.0 with sodium bicarbonate.

Isolation of peripheral blood leukocytes

Heparinized blood was obtained by puncture of the caudal vessel and mixed with an equal volume of carp RPMI (cRPMI; RPMI 1640 adjusted to 270 mosmol kg^{-1} by addition of H_2O) containing 10 i.u. ml^{-1} heparin (Leo Pharmaceutical Products Ltd, Weesp, the Netherlands). After two successive centrifugation steps at 100 ***g*** for 10 min and 700 ***g*** for 5 min, with the brake disengaged and at 4 °C, white cells at the interface of the cell bed were collected and layered on 1.5 volumes of Lymphoprep (density 1.077 g ml^{-1}; Nycomed Pharma, Oslo, Norway). Following subsequent centrifugation at 800 ***g*** for 25 min and at 4 °C, the leukocyte cell layer at the interface was collected and washed three times with cRPMI. The cells were resuspended in cRPMI at a final density of 10^7 cells ml^{-1}.

Immunocharacterization of peripheral blood leukocytes

Peripheral blood leukocytes (PBL) (1.25×10^6 cells ml^{-1}) were incubated for 30 min at 4 °C with a monoclonal antibody (mAb) against carp Ig H chain (WCI-12) (Secombes *et al.* 1983; Rombout *et al.* 1990), carp monocytes/macrophages/thrombocytes (WCL-15) (Weyts *et al.* 1997*a*), or thrombocytes (WCL-6) (Rombout *et al.* 1996). Cells were centrifuged at 680 ***g*** for 7 min at 4 °C, resuspended in cRPMI, and incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (RAM-Ig) antibody (1:100; Dako A/S, Glostrup, Denmark) for 20 min at 0 °C. After washing, 10 000 cells were analysed using a fluorescence-activated cell sorter (FACStar) (Becton Dickonson, Mountain View, CA, USA) tuned at 488 nm using DataMATE software (Applied Cytometry Systems, Sheffield, UK) (see Fig. 1A). The percentage of positive cells within the lymphocyte gate was determined (Koumans-van Diepen *et al.* 1994).

Loading of lymphocytes with Fluo-3/AM or Fura-2/AM

Lymphocytes (10^7 cells ml^{-1}) were loaded with fluorescent Ca^{2+} indicator at room temperature and in the dark. Fluo-3/AM (Sigma, St Louis, MO, USA) (Minta and Tsien, 1989) or Fura-2/AM (Sigma) (Tsien *et al.* 1982*a*), stored as 1 mmol l^{-1} stock solutions in dry dimethylsulphoxide (DMSO), was added at a final concentration of 4 μmol l^{-1}. To improve loading efficiency, 6 μl of Pluronic F-127 (30 % w/v in cRPMI; Sigma) was included in the loading medium. After 40 min, the loading solution was diluted 1:10 with cRPMI and, after incubation for another 10 min, the cells were collected by centrifugation for 7 min at 700 ***g***. The pellet was resuspended in cRPMI at a density of 1.25×10^6 cells ml^{-1}. Cells were then incubated at 26 °C before and during $[Ca^{2+}]_i$ analysis.

Flow cytometric analysis of Fluo-3-loaded cells

Fluorescence emission from Fluo-3/loaded cells was monitored at 530±30 nm by flow cytometry, as reported by Vandenberghe and Ceuppens (1990). The fluorescence in each sample was established at 2.5 or 4 min intervals (experimental samples were measured in parallel at 30 s intervals). After addition of the stimulus, fluorescence intensity was reassessed over 10 s periods every 2.5 or 4 min thereafter. Where indicated, EDTA (10 mmol l^{-1}, Sigma), to chelate extracellular Ca^{2+} (1 mmol l^{-1}), or thapsigargin (10 μg ml^{-1}, Sigma), to prevent Ca^{2+} uptake into intracellular stores, was added. To convert the arbitrary fluorescence units, which may vary in each experiment owing to differences in Fluo-3 loading, into absolute $[Ca^{2+}]_i$, the calibration procedure described by Tsien *et al.* (1982*b*) was applied:

$$[Ca^{2+}]_i = K_d \times [F - F_{min}]/[F_{max} - F]\,. \quad (1)$$

A dissociation constant (K_d) for the Fluo-3/Ca^{2+} complex of 400 nmol l^{-1} was used (Minta and Tsien, 1989). F_{max} represents the maximum fluorescence, i.e. the fluorescence of cells with Ca^{2+}-saturated Fluo-3. Apparent Fluo-3 saturation was achieved by adding 10 mmol l^{-1} bromo-A23187, a non-fluorescing derivative of the Ca^{2+} ionophore A23187 (Sigma), in medium containing 1 mmol l^{-1} Ca^{2+}. F represents the fluorescence of the experimental sample. F_{min} was obtained by adding 1.2 mmol l^{-1} $MnCl_2$ to cells exposed to Ca^{2+} ionophore. Mn^{2+} displaces Ca^{2+} from Fluo-3 and forms a complex that is eight times more fluorescent than the Mn^{2+}-free dye, thus giving 20 % of the fluorescence of the Fluo-3/Ca^{2+} complex. F_{min} is calculated as:

$$F_{min} = F_{max} - (F_{max} - F_{MnCl_2}) \times 1.25\,. \quad (2)$$

Digital imaging microscopy of Fura-2-loaded cells

For $[Ca^{2+}]_i$ measurements in single lymphocytes, Fura-2-loaded cells were allowed to adhere to a poly-L-lysine (Sigma) coated glass coverslip for 45 min at room temperature. The coverslip, forming the bottom of an incubation chamber, was subsequently rinsed three times with cRPMI and filled with 500 µl of cRPMI. The chamber was placed on the stage of an inverted microscope (Nikon Diaphot). The light from a 100 W xenon lamp was directed through a quartz neutral-density filter (Ealing Electro-Optics, Holliston, MA, USA) with a density of 1.5 or greater to avoid damage to the two excitation filters and to reduce bleaching of the intracellularly trapped fluorochrome. The excitation filters, mounted in a motor-driven rotating filter wheel, were bandpass filters with transmission maxima at 340±12 and 380±12 nm (Ealing Electro-Optics). Fura-2 fluorescence was recorded at 492 nm by means of an intensified charge-coupled device (CCD) camera (Photonic Science) operating at video frame rate with the filter wheel in the discontinuous stepping mode. An epifluorescent ×40 magnification immersion objective was used to allow simultaneous monitoring of an average of approximately 60 individual lymphocytes. Dynamic video imaging was carried out using MagiCal hardware and TARDIS software provided by Joyce Loebl (Dukesway, Team Valley, Gateshead, Tyne and Wear, UK) as described previously (Willems *et al.* 1993). The fluorescence emission ratio at 492 nm was monitored as a measure of $[Ca^{2+}]_i$ after excitation at 340 and 380 nm (Nitschke *et al.* 1991). The interframe interval between the ratio frames was 6 s.

Results

Characterisation of peripheral blood leukocytes

The percentage of Ig^+ cells within the 'lymphocyte gate', as determined using the anti-carp-Ig monoclonal antibody WCI-12, amounted to 48 %, whereas the percentage of thrombocytes, as determined using monoclonal antibody WCL-6, was 15 %. A positive reaction with monoclonal antibody WCL-15 was observed with 37 % of the cells in the lymphocyte gate.

$[Ca^{2+}]_i$ in non-stimulated peripheral blood leukocytes

Flow cytometric measurement of Fluo-3 fluorescence from non-stimulated PBLs revealed a sharply peaked distribution, indicating homogeneous dye loading in the different cells of the mixed population (Fig. 1B). From the fluorescence intensity obtained, the resting $[Ca^{2+}]_i$ was calculated to be 150±25 nmol l^{-1} (mean ± S.D., N=3). Chelating extracellular Ca^{2+} (1 mmol l^{-1}) with EDTA (10 mmol l^{-1}) resulted in a slight decrease in baseline fluorescence, whereas thapsigargin (10 µg ml^{-1}), a specific inhibitor of sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPase activity, induced a marked rise in $[Ca^{2+}]_i$, which lasted at least 30 min (Fig. 1C).

Digital imaging microscopy of non-stimulated Fura-2-loaded PBLs demonstrated the presence of both oscillating and non-oscillating cells. Oscillating cells displayed $[Ca^{2+}]$ oscillation patterns that varied from markedly regular to highly irregular (see Figs 3, 5). In addition, oscillating cells displayed a large heterogeneity with respect to the frequency and amplitude of the oscillatory changes in $[Ca^{2+}]_i$.

PHA-induced changes in $[Ca^{2+}]_i$ in peripheral blood leukocytes

Fluo-3-loaded cells responded to PHA with a concentration-dependent increase in fluorescence intensity (Fig. 2). This increase was attributed to 35±3 % (mean ± S.D.) of the PBLs. $[Ca^{2+}]_i$ rose within 10 s following the onset of stimulation. Measurements performed in the presence of EDTA, to achieve nominally Ca^{2+}-free medium, demonstrated that the initial rise in $[Ca^{2+}]_i$ was independent of extracellular Ca^{2+} (Fig. 1C). By contrast, the sustained increase in $[Ca^{2+}]_i$, which lasted for at least 30 min, was abolished in the presence of EDTA (Figs 1C, 2), demonstrating its dependence on the presence of extracellular Ca^{2+}.

Monitoring the changes in fluorescence emission ratio (340 nm/380 nm) in single, Fura-2-loaded cells using digital imaging microscopy, revealed significant PHA-induced changes in $[Ca^{2+}]_i$ dynamics in one-third of the cell population. Responding cells consisted of both oscillating cells and cells that did not display $[Ca^{2+}]_i$ oscillations in the resting situation. Both cell types showed a rapid PHA-induced increase in $[Ca^{2+}]_i$, which, in most cases, was followed by oscillatory changes in $[Ca^{2+}]_i$ originating from an increased baseline level. Cells that displayed $[Ca^{2+}]_i$ oscillations in the absence of any added stimulus, but which reacted to PHA with an increase in baseline $[Ca^{2+}]_i$, in many cases showed an increase in oscillation frequency and amplitude (Fig. 3).

Changes in $[Ca^{2+}]_i$ in PBL following anti-Ig cross-linking or stimulation with LPS

Treatment of PBL with LPS did not change the fluorescence emission intensity as recorded from Fluo-3-loaded cells (N=5). This observation demonstrates that LPS does not increase $[Ca^{2+}]_i$ in carp lymphocytes (Fig. 2).

Cross-linking of Ig with WCI-12 and RAM-Ig resulted in a marked increase in $[Ca^{2+}]_i$ in 45±3 % (mean ± S.D., N=5) of the cells (Fig. 4). The onset of the increase in $[Ca^{2+}]_i$ occurred within 10 s after the addition of the cross-linking RAM-Ig. The peak value of the increase in $[Ca^{2+}]_i$ depended on the concentration of WCI-12. At each WCI-12 concentration, the responding cells formed a sharp peak. This finding demonstrates that the peak increase in $[Ca^{2+}]_i$ is remarkably constant between individual cells. At high concentrations, WCI-12 increased $[Ca^{2+}]_i$ without the addition of RAM-Ig. RAM-Ig alone induced a slight increase in $[Ca^{2+}]_i$ in cells that were not treated with WCI-12 (Fig. 4). Addition of EDTA at the end of an experiment abolished the cross-linking-induced increase in fluorescence. Simultaneous addition of EDTA and WCI-12+RAM-Ig evoked an initial peak of fluorescence that was abolished within 2.5 min.

Digital imaging microscopy revealed that both non-oscillating and oscillating cells responded to cross-linking. In

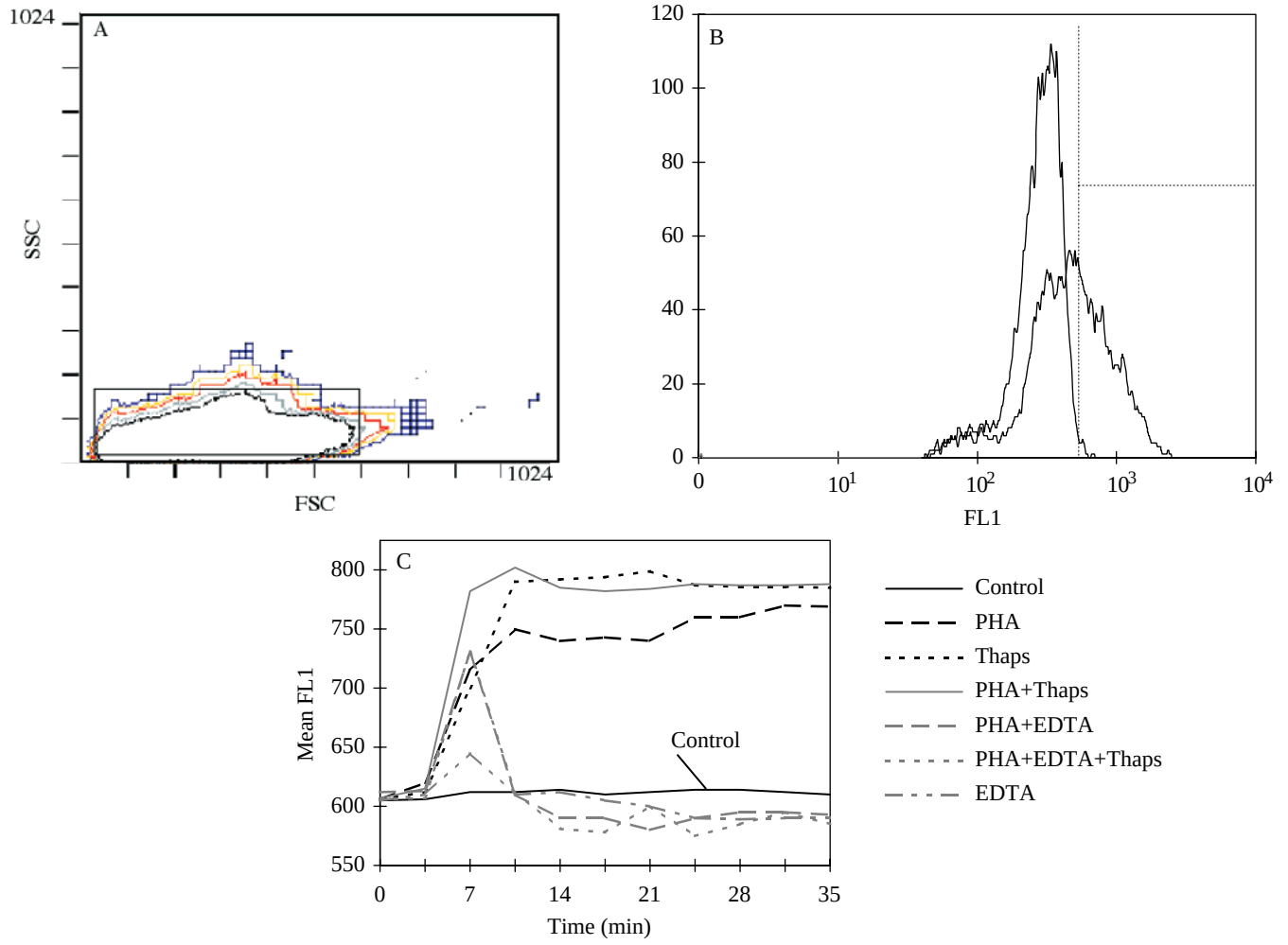

Fig. 1. (A) Forward–sideward scatter (FSC/SSC) dot-plot of peripheral blood leucocytes (PBL) for 10 000 cells. The box indicates the setting for the lymphocyte gate that was used throughout the experiments. (B) Fluorescence measurement (FL1) in Fluo-3-loaded PBLs before (bold line) and after (faint line) stimulation with 50 μg ml^{-1} phytohaemagglutinin (PHA). The vertical line indicates the line set for calculation of the percentage of non-reacting *versus* the percentage of reacting cells. The reacting population consists of 35 % of the total number of PBL. (C) Fluorescence measurements (FL1) in Fluo-3-loaded non-stimulated and PHA-stimulated (50 μg ml^{-1}) PBLs with and without the addition of thapsigargin (Thaps, 10 μg ml^{-1}) and EDTA (10 mmol l^{-1}). Agents were added after 8 min, and FL1 was recorded 10 s later.

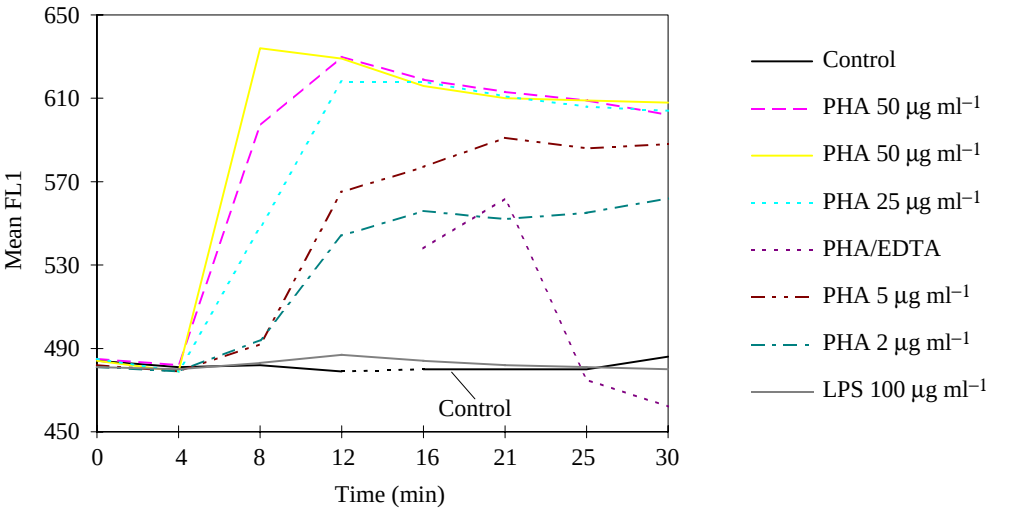

Fig. 2. Changes in mean fluorescence intensity (FL1) in Fluo-3-loaded peripheral blood leucocytes (PBL) after stimulation with 2, 5, 25 and 50 μg ml^{-1} phytohaemagglutinin (PHA), with 100 μg ml^{-1} lipopolysaccharide (LPS) and with 50 μg ml^{-1} PHA in nominally Ca^{2+}-free medium (PHA/EDTA); EDTA was added at 16 min. PHA or LPS was added after 8 min and FL1 was recorded 10 s later.

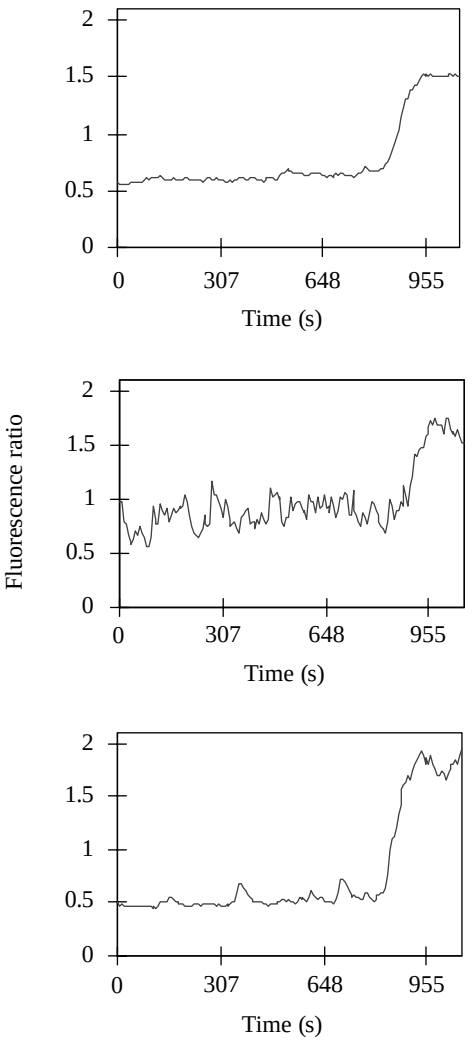

Fig. 3. Measurement of Ca^{2+} oscillations from the fluorescence ratio at 340 nm/380 nm in three single Fura-2-loaded cells by digital imaging microscopy prior to and after stimulation with 50 μg ml^{-1} phytohaemagglutinin (PHA) at 850 s.

most cases, non-oscillating cells were found to respond with oscillatory changes in $[Ca^{2+}]_i$ (Fig. 5C). Oscillating cells mostly responded with a significant increase in oscillation frequency (Fig. 5B). The percentage of responding cells was estimated to be 40–50 %.

Discussion

This work clearly demonstrates that carp lymphocytes respond with an increase in $[Ca^{2+}]_i$ upon stimulation with T-cell mitogen PHA and membrane Ig cross-linking with anti-Ig Mab. However, carp lymphocytes did not display any increase in $[Ca^{2+}]_i$ upon stimulation with LPS. In the presence of both stimuli, the initial increase in $[Ca^{2+}]_i$ was independent of the presence of external Ca^{2+}, showing that the receptors involved are coupled to the increased production of InsP_3. The present findings indicate an important role for Ca^{2+} in regulating the cellular activity of lymphocytes and an evolutionarily well-conserved mechanism of intracellular Ca^{2+} signalling.

A great advantage of flow cytometry for measurement of the cytosolic free Ca^{2+} concentration is that only intact cells are analysed, because any non-vital cells and cell debris are gated out. Moreover, gating and double-staining techniques enable the analysis of selected subpopulations of PBLs.

The mean value for $[Ca^{2+}]_i$ in resting carp lymphocytes was calculated to be 150 nmol l^{-1}, which is close to the value obtained with mammalian lymphocytes using either Quin-2 or Indo-1 as Ca^{2+} indicator (Vandenberghe and Ceuppens, 1990). Similar values have also been reported for tilapia chloride cells and the pavement cells of the operculum epithelium (Li *et al.* 1997) and are in fact common for most vertebrate cell types.

The Ca^{2+} fluctuations within individual cells show striking similarity in oscillation profile with those of mammalian lymphocytes, suggesting a similar biphasic feedback mechanism of cytosolic $[Ca^{2+}]$ on InsP_3-induced Ca^{2+} release, i.e. relatively low cytosolic $[Ca^{2+}]$ enhancing InsP_3-induced Ca^{2+} release, and relatively high cytosolic $[Ca^{2+}]$ inhibiting this process (Finch *et al.* 1991; Clapham and Sneyd, 1995; Verheugen and Vijverberg, 1995).

The present study shows that PHA can induce a rapid increase in $[Ca^{2+}]_i$ in a subpopulation of carp PBLs. In mammals, PHA has been demonstrated to react with the T-cell receptor to induce a rapid rise in $[Ca^{2+}]_i$ (Tsien *et al.* 1982*a*,*b*; Sei and Aurora, 1991; Vandenberghe and Ceuppens, 1990).

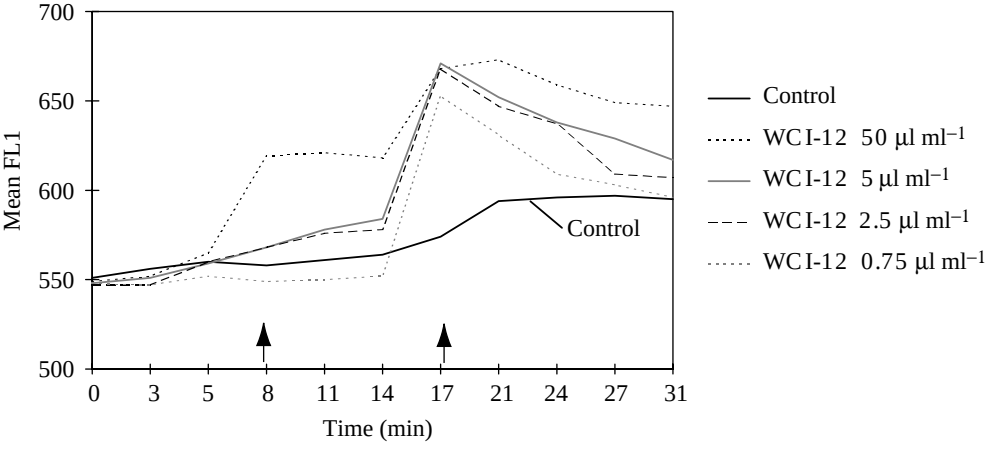

Fig. 4. Mean fluorescence intensity (FL1) of Fluo-3-loaded peripheral blood leucocytes (PBL) in the absence and presence of the monoclonal antibody WCI-12 (0.75, 2.5, 5 and 50 μl ml^{-1}). Rabbit-anti-mouse-Ig (RAM-Ig, 100 μl ml^{-1}) was added to all samples. An arrow indicates addition of WCI-12 (at 7 min 50 s) and addition of RAM-Ig (17 min 50 s).

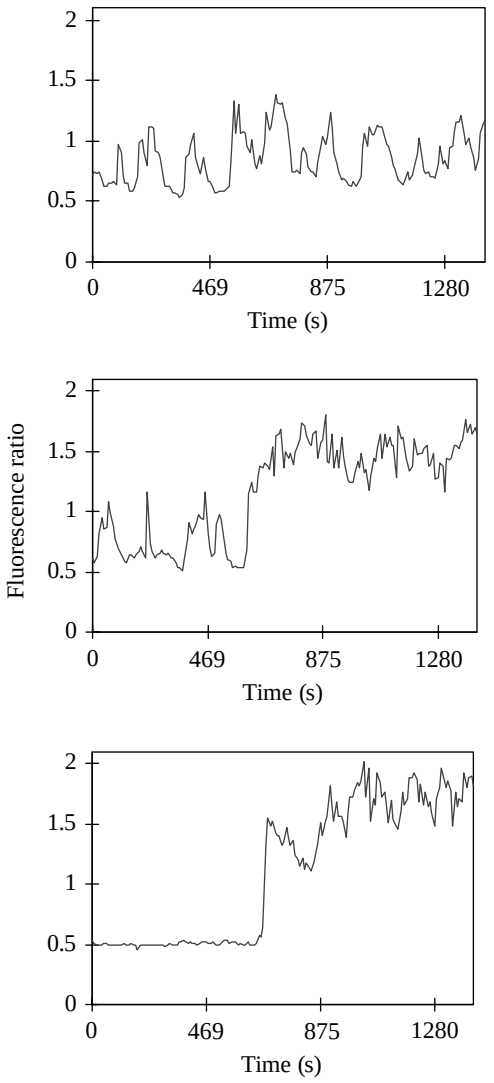

Fig. 5. Measurement of Ca^{2+} oscillations from the fluorescence ratio at 340 nm/380 nm in three single Fura-2-loaded cells by digital imaging microscopy prior to and after stimulation with WCI-12 plus RAM-Ig (25 µl ml^{-1} and 100 µl ml^{-1}, respectively) at 500 s.

Although, in fish, separation of T-cells within the Ig^- population has not yet been achieved, PHA has been shown preferentially to induce proliferation in cells from the Ig^- population of carp PBL (Koumans-van Diepen *et al.* 1994), indicating that lectins are also good T-cell stimulators in fish. The initial rise in $[Ca^{2+}]_i$ in response to stimulation with PHA was independent of the presence of extracellular Ca^{2+} and must therefore be due to Ca^{2+} release from intracellular InsP_3-sensitive stores. In contrast, the sustained plateau response was eliminated in nominally Ca^{2+}-free medium and thus depends on the influx of Ca^{2+}. This biphasic response is similar to that observed in T-cells of several mammalian species (Vandenberghe and Ceuppens, 1990) and therefore seems to be an evolutionarily well-conserved phenomenon.

Single-cell measurements revealed that a proportion of the carp lymphocytes displayed spontaneous $[Ca^{2+}]_i$ oscillations when incubated in the absence of any stimulus. However, addition of PHA clearly affected both oscillating and non-oscillating cells, and the percentage of responding cells was calculated to be 30 %. To the best of our knowledge, this is the first report of $[Ca^{2+}]_i$ oscillations in lymphocytes from a lower vertebrate. Carp lymphocytes displayed a large heterogeneity in response pattern upon stimulation with PHA, varying from single transient increases to sustained elevations and oscillatory changes in $[Ca^{2+}]_i$. A similar observation was made with mammalian lymphocytes (Sei and Arora, 1991). In mammals, the precise amplitude and frequency of cytoplasmic Ca^{2+} oscillations is considered to be essential for modulating the stimulatory signal to induce further events associated with activation, e.g. IL-2 synthesis (Zmonarski *et al.* 1996). Moreover, different stimuli have been shown to evoke different patterns of cytosolic Ca^{2+} signalling (Spinozzi *et al.* 1995*a*). The occurrence of different oscillation patterns may well be due to the presence of different lymphocyte subtypes or to differences in the maturation or activation state of the cells.

The mechanism of B-lymphocyte activation in fish is still largely unknown. For carp, LPS was shown to be a selective stimulator of Ig^+ lymphocytes. LPS has been shown to induce a substantial increase in cell proliferation (Weyts *et al.* 1997*b*), but the present study demonstrates that it fails to increase $[Ca^{2+}]_i$. This is again comparable with the situation in mammals and in agreement with observations in catfish, the only other fish species studied in this respect (van Ginkel *et al.* 1994). Cross-linking of putative surface Ig on B-lymphocytes with WCI-12 and RAM-Ig induced a rapid increase in $[Ca^{2+}]_i$. The profile of stimulation in catfish, as in mammals, consisted of an initial peak followed by an extracellular-Ca^{2+}-dependent sustained plateau. This cytosolic Ca^{2+} signal may represent the intracellular signal for DNA synthesis and cell proliferation, as can be concluded from the observation that catfish lymphocytes respond to Ig cross-linking with increased thymidine incorporation (van Ginkel *et al.* 1994). However, the present finding that LPS stimulation, in contrast to WCI-12 cross-linking, does not lead to an increase in $[Ca^{2+}]_i$, demonstrates that the two stimuli act through different intracellular pathways. Similar results have been obtained with mammalian B-cells (Sei and Arora, 1991; Bijsterbos *et al.* 1985).

The mechanism of B-cell stimulation is now starting to be unravelled for mammals, in which activation of B-cells *via* the Ig receptor is known to be coupled to the phospholipase-C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate inositol 1,4,5-trisphosphate and diacylglycerol, which serve as intracellular messengers for the generation of intracellular Ca^{2+} signals and protein phosphorylation by protein kinase C. This has been confirmed in lower vertebrates only for the channel catfish (van Ginkel *et al.* 1994). Release of intracellular Ca^{2+} through a thapsigargin-sensitive pathway after triggering by Ig-crosslinking indicates that the Ig receptor is also coupled to the activation of phospholipase C in carp lymphocytes.

In fish, the immunoglobulins are of an IgM isotype (Wilson and Warr, 1992). Induction of cell proliferation in response to

Ig cross-linking is found in mature mammalian IgM- and IgD-co-expressing lymphocytes. This, however, appears to be due to IgD cross-linking, whereas IgM cross-linking has been associated with apoptosis (Grandien *et al.* 1994; Graves *et al.* 1996). Also, immature IgM-expressing B-cells undergo inhibition of proliferation after cross-linking (Grandien *et al.* 1994). In contrast, catfish leukocytes were reported to proliferate after Ig-crosslinking (van Ginkel *et al.* 1994). Since the positive *versus* negative effect on proliferation may be affected by antigen concentration and valency, by the duration of B-cell receptor occupancy and affinity and by the differentiation stage of the B cell (Grandien *et al.* 1994; Galibert *et al.* 1996), it is too early to draw conclusions on the physiological effects of Ig receptor cross-linking in fish.

The present study shows that the percentage of WCI-12-immunoreactive lymphocytes (48 %), representing the Ig^+ cells, is in good agreement with the percentage of cells displaying an increase in $[Ca^{2+}]_i$ upon cross-linking (45 %). This finding demonstrates that the whole 'B'-lymphocyte population responds to cross-linking with an increase in $[Ca^{2+}]_i$.

Measurements of $[Ca^{2+}]_i$ changes in single cells or specific cell populations will provide not only insights into signal transduction processes in fish leukocyte (sub)types but also an accurate tool for the measurement of phospholipase-C-induced cell activation. This may be of great importance to toxicology, especially since the early stages of T-cell activation are considered to be a possible site of action of various pathological and toxicological factors (Manzo *et al.* 1996). Sustained high levels of $[Ca^{2+}]_i$ will induce T-lymphocytes to enter apoptosis, with consequent impact on the immunological functioning of the animal (Nicotera *et al.* 1994; Spinozzi *et al.* 1995*b*).

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