Response of PAS-positive cells of the pituitary pars intermedia in the teleost *Carassius auratus* to acid water

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**Summary.** The PAS-positive or PIPAS cells in the pars intermedia of goldfish are activated after reduction of the pH of the ambient freshwater from 7.5 to 3.5. The cells increase in number and exhibit a five-fold increase in cell volume. Granular endoplasmic reticulum occupies most of the cytoplasm. Goldfish PIPAS cells (also termed calcium-sensitive cells) are thought to have a hypercalcemic function. Therefore, their activation in acid water may be caused by the severe drop in plasma calcium concentration following exposure of the fish to low water pH. However, activation of the PIPAS cells in response to acidification of the water is not prevented when the calcium concentration of the water is increased to levels that result in hypercalcemia instead of hypocalcemia. Activation of the PIPAS cells occurs also in fish exposed to acidified freshwater enriched with NaCl to an osmolarity similar to that of the blood. This prevents the reduction in plasma osmolarity and Na⁺ and Cl⁻ concentrations that follow exposure of goldfish to acidified normal freshwater. Our observations do not support the hypothesis that the PIPAS cells in goldfish produce a hypercalcemic hormone, or indeed any hormone involved in calcium metabolism or osmoregulation. The cells may be implicated in acid-base regulation (a characteristic of many types of fish when exposed to acidified water) but the evidence is indirect.

**Key words:** Pituitary gland, pars intermedia (Teleosts) – PAS-positive cells – Acidification of water – Calcium regulation

Acidification of the water leads to reduction of fish populations, diminished diversity of fish species and, finally, disruption of the aquatic ecosystem (Harvey 1980). Osmoregulatory disturbances, mainly due to increased ion losses through gills and kidneys, are considered an important cause of the deleterious effects of acid water (Muniz and Leivistad 1980; McDonald and Wood 1981; McDonald 1983a, b; Wendelaar Bonga et al. 1984a). In line with this conclusion are observations that exposure to acid water induces enhanced secretion of typically osmoregulatory hormones, like cortisol and prolactin (Ashcom 1979; Wendelaar Bonga et al. 1984a, b). It is likely that these hormones are involved in the restoration of water and ion metabolism during adaptation to acid stress. The present paper forms part of our studies on the effect of acid water on the endocrine system, in particular osmoregulatory hormones, in teleost fish.

In an earlier study on goldfish (*Carassius auratus*) and the African cichlid fish *Oreochromis mossambicus* we have found differences in the response of the pituitary gland of the two species to acidification of the water. In *O. mossambicus* the prolactin cells became highly activated. However, no changes could be observed in the prolactin cells of goldfish (Wendelaar Bonga et al. 1984a, b). Contrastingly, in goldfish the PAS-positive cells of the pars intermedia (further termed PIPAS cells) became dramatically activated during exposure to acid water (Wendelaar Bonga et al. 1984b). PIPAS cells are exclusively found in teleosts (Ball and Batten 1981). Their function is still in dispute (Ball and Batten 1981; Pang 1981; Ball et al. 1982; Olivereau et al. 1982a, b; Van Eys and Wendelaar Bonga 1984). In some fish species these cells respond to changes in osmolarity of the ambient water, or to changes in background reflectivity (Ball and Batten 1981; Van Eys 1980). In the killifish (*Fundulus heteroclitus*; Ball et al. 1982), eels (*Anguilla anguilla*) and goldfish (Olivereau et al. 1980a, b) the PIPAS-cells increase markedly in size and number when fish are exposed to calcium-deficient water. This response can be prevented by supplying calcium ions to the water, and therefore the PIPAS cells have been denoted as calcium-sensitive cells in goldfish and eels (Olivereau et al. 1980b; Olivereau and Olivereau 1982). It has been suggested that they are the source of a hypercalcemic hormone comparable to prolactin in other fish species (Olivereau et al. 1980; Pang 1981).

Our initial observations on the effect of low water-pH on the PIPAS cells in goldfish were consistent with this hypothesis (Wendelaar Bonga et al. 1984b). The activation of these cells, observed during the first two weeks of exposure of the fish to acid water may represent an endocrine response to restore the marked drop in plasma calcium that immediately follows a reduction in water pH. In the present study we have further explored the relationship between the PIPAS cells and calcium, by using acidification of water as a tool to manipulate plasma ion levels. The results raise doubts about the involvement of the PIPAS cells in the endocrine control of plasma calcium.
Materials and methods

Sexually immature goldfish (Carassius auratus) weighing 15-20 g were purchased from a commercial fish dealer. The fish were kept in well-aerated glass aquaria (8 fish per 100 l) at 20° C and a daily light period of 12 h. Four weeks before acidification of the water, two groups of 8 fish were adapted to high-calcium freshwater (CaCl₂ in tap water) and two further groups of 8 fish to saline (NaCl in tap water). The composition of the water used has been reported earlier (Wendelaar Bonga et al. 1984b). The Ca²⁺ concentration was 0.8 mM, the Na⁺ concentration 1.9 mM and the pH 7.5. The CaCl₂ and NaCl concentrations of the water were increased stepwise by adding 20% of the final concentrations every third day. CaCl₂ was added to a final Ca²⁺ concentration of 6 mM, and NaCl to a final osmolarity of 300 mOsmol/l.

The fish were exposed to acid water for two weeks. On the first day (day 0), the pH of the water was reduced from 7.5 to 4.5 by adding H₂SO₄ (analytical grade). On days 2 and 4 the pH was reduced to 4.0 and 3.5, respectively. The pH was monitored daily and adjusted by titration with H₂SO₄. The water was replaced every third day to keep nitrogenous wastes at a low level. During the experiments, with the exception of the first three days, the control fish (3 groups kept in tap water, saline or high-calcium tap water at pH 7.5) and the acid-exposed fish (3 groups kept under the same conditions except for pH) were fed on Tetramin fish food. No deaths occurred during the experiments.

On days 0, 1, 7 and 14, groups of 8 fish were anesthetized in 0.2% methoxyethanol (dissolved in the water the fish had been adapted). Blood was collected from the cut end of the caudal peduncle and plasma was collected by centrifugation in heparinized microhematocrit capillaries. Plasma osmolarity and calcium levels were determined as described (Wendelaar Bonga et al. 1984b). Plasma Na⁺ and Cl⁻ were determined by atomic absorption spectrophotometry.

The pituitary glands of the fish killed by day 14 were excised and prepared for electron microscopy. Methods used for fixation and embedding, for the determination of cellular and nuclear areas, and for statistical analysis, were the same as described previously (Wendelaar Bonga et al. 1984b).

Results

1. PIPAS cells

The pars intermedia of goldfish contains two types of endocrine cells, the cells that produce the melanophore stimulating hormone (MSH) and the PIPAS cells, which are interspersed by non-secretory stellate cells. The PIPAS cells are concentrated in those areas of the pars intermedia that border on the neural part of the pituitary gland. The pars intermedia of the goldfish has been previously studied using light and electron microscopy (Leatherland 1972; Olivereau et al. 1980b; Wendelaar Bonga et al. 1984b).

Fish from water at pH 7.5. In goldfish from normal freshwater (Ca²⁺:0.8 mM; 8 mOsmol/l), the PIPAS cells are small and low in number. They occur singly or in groups of two or three cells. The cytoplasm shows many electron-dense secretory granules, isolated strands of granular endoplasmic reticulum, some mitochondria and usually a small Golgi area (Figs. 1, 2). In fish adapted for 6 weeks to high-calcium water (6 mM Ca²⁺/l), or to a saline solution with an osmolarity similar to that of the blood plasma of freshwater goldfish (300 mOsmol/l), the distribution and ultrastructure of the PIPAS cells is not noticeably changed (Figs. 3, 4). The cell and nuclear areas are similar to those of fish from normal freshwater (Fig. 5).

Fish from water at pH 3.5. In fish exposed for two weeks to acidified normal freshwater, the PIPAS cells show dramatic changes in number and structure. The pars intermedia contains fields that are occupied exclusively by many markedly enlarged PIPAS cells. Cellular and nuclear areas are

Figs. 1-4. PIPAS cells of goldfish; the cells contain electron-dense secretory granules; MSH MSH cells surrounding the PIPAS cell; MSH cells contain secretory granules of varying electron density

Fig. 1. A PIPAS cell of a fish from normal freshwater, pH 7.5. ×10500
Fig. 2. Golgi area (Ga) in a PIPAS cell of a fish from normal freshwater, pH 7.5; p peroxisome. ×18500
Fig. 3. A PIPAS cell of a fish from high-calcium freshwater, pH 7.5. ×10500
Fig. 4. A PIPAS cell of a fish from saline, pH 7.5. ×10500
Fig. 6. PIPAS cells of a goldfish exposed to acidified normal freshwater (0.8 mM Ca\textsuperscript{2+}; pH 3.5) for 14 days; the cells show extensive granular endoplasmic reticulum (er) and few secretory granules. The cell in the centre contains small granules. × 10500

Fig. 7. A PIPAS cell of a goldfish exposed to acidified high-calcium freshwater (6 mM Ca\textsuperscript{2+}; pH 3.5) for 14 days; the cells show extensive granular endoplasmic reticulum (er) and Golgi areas (Ga). × 10500
Fig. 8. A large Golgi area with many presecretory granules (arrows) in a PIPAS cell of a goldfish exposed to acidified high-calcium freshwater (6 mM Ca\(^{2+}\); pH 3.5) for 14 days. \(\times 18500\)

Fig. 9. A PIPAS cell of a goldfish exposed to acidified saline (pH 3.5) for 14 days; the granular endoplasmic reticulum (er) is extensive and secretory granules are scarce. \(\times 10500\)
Table 1. Effect of exposure to water at pH 3.5 for 1, 7 or 14 days on plasma Na⁺ and Cl⁻ concentrations (mM) of goldfish pre-adapted to freshwater or isoosmotic saline at pH 7.5 (Ca²⁺: 0.8 mM). Controls: plasma Na⁺ and Cl⁻ values (blood collected at day 0) of freshwater- and saline-adapted fish at pH 7.5 (Ca²⁺: 0.8 mM). Means ± S.D.; n = 7

<table>
<thead>
<tr>
<th>pH 7.5 (controls)</th>
<th>pH 3.5</th>
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<tr>
<td></td>
<td>day 1</td>
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<tr>
<td>Freshwater</td>
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<tr>
<td>Na⁺</td>
<td>153.3 ± 7.4</td>
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<tr>
<td>Cl⁻</td>
<td>125.7 ± 8.4</td>
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<tr>
<td>Saline</td>
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<tr>
<td>Na⁺</td>
<td>166.6 ± 9.7</td>
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<tr>
<td>Cl⁻</td>
<td>140.4 ± 6.4</td>
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a Significantly different from freshwater controls, P<0.05
b Significantly different from freshwater controls, P<0.01
c Significantly different from corresponding values of acid-exposed freshwater fish, P<0.01

2. Plasma osmolarity and total plasma calcium

Immediately after reduction of the pH of the water, plasma osmolarity decreases in fish from normal freshwater and high-calcium freshwater. In normal freshwater, the difference is statistically significant (P<0.001) after 1 day, and the reduction persists throughout the experimental period (Fig. 10). This reduction is mainly accounted for by decreases in plasma Na⁺ and Cl⁻ (Table 1). In fish from the high-calcium group, the drop in osmolarity is less severe than in the fish from normal freshwater, although it is highly significant after 7 and 14 days, respectively (Fig. 10; P<0.01).

At pH 7.5, total plasma calcium levels are similar in fish from normal freshwater and in fish from high-calcium water (Fig. 10, day 0). Acidification of the water leads to significant differences in plasma calcium. In fish from normal freshwater, the calcium level is decreased (Fig. 10; P<0.001); in fish from high-calcium water, there is a slight increase (Fig. 10; P<0.05).

When fish are adapted to saline with an osmolarity of 300 mOsmol/l (a value similar to that of the blood plasma in fish from normal freshwater) plasma osmolarity is increased and maintained at a level higher by 10% than that under normal freshwater conditions. This increase is mainly accounted for by Na⁺ and Cl⁻ (Table 1). Acidification of the water affects neither plasma osmolarity (Fig. 11), nor plasma Na⁺ and Cl⁻ levels (Table 1). Total plasma calcium in saline-adapted fish is similar to the value found in fish from normal freshwater. A drop in pH of the saline leads to a slight decrease in total plasma calcium. The difference from the control value is significant for day 1 only (Fig. 11; P<0.05).

Discussion

The PIPAS cells in goldfish respond to acidification of the water by increased secretory activity. This is concluded from the marked increase in number and size of the cells.
the very conspicuous extension of the granular endoplasmic reticulum, the enlarged Golgi areas with many presecretory granules, and the reduction in the number of secretory granules present in the cytoplasm. The latter phenomenon indicates that, even two weeks after reduction of water pH, the rate of synthesis of the granules (probably greatly accelerated) is balanced by the rate of release. We have not been able to report structural signs of granule release by exocytosis; this is probably because of the scarcity of the granules.

The activation of the PIPAS cells following water acidification still occurs after adapting goldfish to conditions that prevent reduction of plasma osmolarity or total plasma calcium levels.

1. PIPAS cells and calcium

In goldfish, eels and killifish (Fundulus heteroclitus) a relationship has been demonstrated between the calcium concentration of the water and the number and size of the PIPAS cells (Olivereneau et al. 1980a, b; Ball et al. 1982). The marked activation of the PIPAS cells that occurs after transfer of goldfish and eels to distilled water (either slightly acid or buffered), or calcium-deficient seawater, can be prevented by adding calcium ions to the water (Olivereneau et al. 1980b, 1981a, b, 1982a, b; Olivereneau and Olivereneau 1982). The present data show that activation of the PIPAS cells in goldfish in response to lower pH is similar in extent to that reported by Olivereneau et al. (1980b) following treatment with distilled water, as judged by cell and nuclear areas. However, the stimulation induced by firm acidification is not prevented by calcium. Whereas reduction of pH in water with a relatively low Ca\(^{2+}\) concentration leads to a reduction of plasma total calcium levels, a similar drop in pH in the presence of 6 mM Ca\(^{2+}\) results in slight hypercalcemia. Thus, the response of the PIPAS cells to low pH occurs independently of the external and internal calcium concentrations. Our previous suggestion, that the effects of acid water on the PIPAS cells in goldfish are mediated by a reduction in plasma calcium levels (Wendelaar Bonga et al. 1984b), is therefore no longer tenable.

We have made an additional observation that mitigates against calcium as a controlling factor in PIPAS cell activity. Adaptation of goldfish to high-calcium water at normal pH does not noticeably influence the PIPAS cells. The concentration used provides a heavy calcium load for the fish, the Ca\(^{2+}\) concentration of the ambient water being more than twice the total plasma calcium concentration, and four times higher than the plasma Ca\(^{2+}\) level (unpublished observation). We conclude from the present observations that neither the calcium concentration of the water, nor the plasma calcium levels are predominating factors in the control of PIPAS cell activity in goldfish.

The question remains as to whether the PIPAS cells produce a hormone involved in the control of calcium metabolism. Parsons et al. (1978) have found evidence for a hypercalcemic parathyroid-like substance in the pituitary gland of eel (A. anguilla) and cod (Gadus morrhua). They conclude that it is different from prolactin and might originate in the pars intermedia. On the basis of these results and the above-mentioned responses of the PIPAS cells in goldfish and killifish to distilled water and calcium-deficient seawater, Olivereneau et al. (1980b, 1981b), Pang (1981), and Ball et al. (1982) have suggested that the PIPAS cells are the source of a hypercalcemic hormone. Our results raise doubts concerning this conclusion as far as goldfish are concerned. Cells producing classical hypocalcemic or hypercalcemic hormones such as calcitonin and parathyroid hormone respond to changes in plasma calcium levels either by activation or inactivation (Munson and Gray 1970; Sherwood et al. 1966). In fish, the calcitonin cells and the hypocalcin-producing type-I cells of the Stanniustus corpuscles are activated in high-calcium water (Meats et al. 1978; Wendelaar Bonga et al. 1976). Such responses are not consistently found in PIPAS cells. For the cichlid fish Oreochromis mossambicus we have shown that these cells do not respond to changes in external or internal calcium concentrations and we have concluded that in this species the PIPAS cells are unlikely to be involved in the endocrine control of calcium metabolism (Van Eys and Wendelaar Bonga 1984). The present data on goldfish support this conclusion. Although PIPAS-cell activity in goldfish may be correlated with external calcium levels under particular experimental conditions of acid stress, the name "calcium-sensitive cells" (as has been proposed; Olivereneau et al. 1980b) is inadequate.

2. PIPAS cells and osmolarity

If goldfish are exposed for a short time to acidified normal freshwater, plasma osmolarity decreases rapidly. A similar reduction has been reported for acid-treated O. mossambicus (Wendelaar Bonga et al. 1984a, b) and rainbow trout (Salmo gairdneri, McDonald 1983a); this can be mainly attributed to losses of Na\(^+\) and Cl\(^-\). Our present data show that in goldfish the drop in osmolarity and Na\(^+\) and Cl\(^-\) levels is prevented when the fish have been adapted to a saline solution with an osmolarity similar to that of the plasma of freshwater goldfish. Since the activation of the PIPAS cells after acidification of the water is not prevented by saline treatment, we conclude that the effects of low water pH on these cells are not mediated by changes in plasma osmolarity, Na\(^+\) or Cl\(^-\). This is in line with the observation of Olivereneau et al. (1981b, 1982a) that the stimulation of PIPAS cells in goldfish following transfer to de-mineralised water is unrelated either to external or internal osmolarity or to external Na\(^+\) and Cl\(^-\) concentrations, and suggests that PIPAS cells play no role in osmoregulation or regulation of monovalent ions.

3. PIPAS cells and water pH

The PIPAS cells in goldfish are highly activated in water of low pH, irrespective of the osmolarity and calcium concentration. This response is difficult to interpret, since the effects of acid water on fish are manifold, complicated, and incompletely explored. Structural skin damage, increased mucification of skin and gills (Daye and Garside 1976), increased permeability of skin and gills to water and ions (McWilliams 1982; McDonald 1983a; Wendelaar Bonga et al. 1984a), reduced branchial Na\(^+\)-influx (McWilliams 1980; McDonald et al. 1980), decreased bone mineralization and disturbed acid-base balance (Packer 1979; McDonald et al. 1980; Ulltisch et al. 1981) are among the effects that have been reported. Moreover, reduced blood pH and bicarbonate levels (Janssen and Randall 1979; Packer 1979; McDonald et al. 1980), reduced plasma Na\(^+\), Cl\(^-\) and calcium concentrations (McDonald et al. 1980; Wendelaar Bonga et al. 1984a, b), increased urinary losses...
of Na\(^+\), Cl\(^-\), and phosphate (McDonald 1983a), and increased ammonia excretion (Ultsch et al. 1981; McDonald and Wood 1981) have been described. Thus, the observation that the PIPAS cells in goldfish are highly active in acid water does not provide a clear insight as to the function of these cells. As we have concluded above, the response of the PIPAS cells to acidification of the water is probably unconnected with the reduction of plasma osmolarity and Na\(^-\), Cl\(^-\), or calcium levels. It is also unlikely to be connected with the increased permeability of the integument to water and ions, since high calcium levels in water are known to reduce integumental permeability (Potts and Fleming 1971; Ogasawara and Hirano 1984) and do not influence the structure of the PIPAS cells (see above). The inhibitory effect of the addition of calcium on the PIPAS cells of goldfish kept in mildly acid water (Olivereau et al. 1980b) seems in contradiction with our observations. The difference may, however, be connected with water pH. The effects of calcium ions on fish are different depending on the pH of the water. At neutral and mildly acid pH, a high calcium concentration of the water reduces the ion losses across the integument (McWilliams 1982; McDonald 1983a). However, at lower pH, as in our experiments, high calcium levels aggravate the acid-base disturbance induced by low pH (McDonald et al. 1980). Our observations show that the cellular and nuclear areas of PIPAS cells increase at the same extent in the three groups of goldfish kept at pH 3.5, irrespective of the calcium concentration of the water. The Golgi areas, however, are more enlarged and seem more active in fish from high calcium water than in fish from the other two groups. Thus, although the present results are inconclusive regarding the function of the PIPAS cells in goldfish, there is circumstantial evidence for a role in acid-base regulation.

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