Analysis of highly phosphorylated inositols in avian and crocodilian erythrocytes

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Abstract

Both morphological and paleontological characteristics support the hypothesis of a monophyletic origin of crocodilian and avian groups. However, while the erythrocytes of all birds studied to date are reported to contain high levels of inositol pentakisphosphate (InsP₅), which acts as an allosteric effector of hemoglobin, this molecule has not been reported in crocodilian erythrocytes. In this study we compare the highly phosphorylated inositols in crocodilian and avian erythrocytes using a particularly sensitive analytical procedure. Our aim was to obtain new data which might provide further evidence for the monophyletic origin, or otherwise, of crocodiles and birds. We studied three avian and three crocodilian species. The erythrocytes of the three bird species contained low levels of inositol-3,4,5,6-tetrakisphosphate and inositol-1,3,4,6-tetrakisphosphate, thought to be precursors of Ins(1,3,4,5,6)P₅. The crocodilian erythrocytes studied contained Ins(1,3,4,5,6)P₅ and InsP₆ in higher concentrations than those found in mammal erythrocytes and in other more active cells such as macrophages. Our data provide further evidence of the similarity between crocodilian and avian groups and agree with the hypothesis that both groups evolved from a common ancestor. The process by which the function of inositol phosphates changed from that of intracellular signaling to hemoglobin allosteric effector is discussed.

Keywords: Inositol phosphates; Organic phosphates; Hemoglobin; Erythrocyte metabolism; Evolution; Oxygen affinity; Crocodilians; Birds; Pigeon; Sparrow; Chicken

1. Introduction

Vertebrate erythrocytes contain high levels of a number of organic phosphates [mainly ATP, GTP, inositol pentakisphosphate (InsP₅) and 2,3-P₂-glycerate (BPG)] that bind to the β-chains of the hemoglobin tetramer, thereby markedly reducing hemoglobin oxygen affinity. Thus, oxygen unloading at moderate $P_{O_2}$ increases, facilitating oxygen release to tissues. Each vertebrate group is associated with the synthesis of particular organophosphates, e.g. BPG in mammals and InsP₅ in birds (see recent discussions of erythrocyte phosphate distribution in vertebrates in Val, 2000; Schweitzer and Marshall, 2001). The ancestral effector molecule is generally accepted to be ATP. This molecule is the hemoglobin allosteric effector in many fish (Bartlett, 1980; Isaacks and Harkness, 1980; Val, 2000). It is also found in the erythrocytes of early tetrapod embryos and continues to be found...
at high levels in many adults. The only allosteric effector not related to the major erythrocyte metabolic pathways is InsP$_3$. It is scarce in fish; to date, inositol phosphates have only been found in the erythrocytes of three fish, one air-breathing fish *Arapaima gigas* (Isaacks et al., 1977) and two elasmobranch fish (Borgese and Nagel, 1978). In tetrapods, InsP$_3$ is present in some reptilian groups (e.g. turtles) and in all birds studied to date, but it is not found in mammals (Rapoport and Guest, 1941; Bartlett, 1980; Isaacks and Harkness, 1980; Riera et al., 1983; Palacios et al., 1984). InsP$_3$ is the alternative hemoglobin effector to BPG of mammal erythrocytes. Bird embryo erythrocytes contain BPG, which, after birth, is replaced by InsP$_3$ (Isaacks and Harkness, 1980). The functional advantage of this molecule in adult bird erythrocytes is not known. In a previous study in anemic quail (Riera et al., 1991), we hypothesized that InsP$_3$ is a better effector than BPG in maintaining constant blood oxygen affinity in birds. How this inositol has become so widespread among birds, however, remains unknown.

Crocodilians are the closest living relatives of the extinct reptile forerunners of birds (Wheatstone and Martin, 1979) and are thought to have remained unchanged throughout recent evolution. In morphological and paleontological terms, crocodiles belong to the group known as Archosauromorpha (Crocodylia, Aves and related extinct forms) which in turn belongs to the Amniota (i.e. reptiles, birds and mammals) (Gorr et al., 1998). Several recent studies have demonstrated functional and behavioral homology between birds and crocodilians, e.g. expression of B-keratins (Sawyer et al., 2000) and patterns of parental care (Tullberg et al., 2002), though such homology is not evident in the analysis of hemoglobin sequences (Gorr et al., 1998). Moreover, other characteristics have been found to be closer to those of mammals (Kalman and Pritz, 2001; Pfatzack et al., 2002). However, crocodilians modulate oxygen affinity by means of bicarbonate (Bauer et al., 1981) and not via any of the organic phosphates present in birds or mammals. In fact, the levels of organic phosphates in adult crocodilian erythrocytes have been considered negligible (Bartlett, 1980; Grigg et al., 1993).

The aim of this study was to obtain precise information about InsP levels and synthesis in crocodilian and avian red blood cells in order to establish possible evolutionary relations between both groups with regard to inositol metabolism.

### 2. Materials and methods

The crocodilians studied were from Barcelona Zoo. Erythrocytes from 6-year-old males of three species were analyzed: *Osteolaemus tetraspis*, *Caiman latirostris* and *Caiman crocodylus*. Blood was drawn in from the retroorbital sinus in anesthetized animals. The resulting packed-cell volume was 18–24%. Urban pigeons (*Columba livia*) from Barcelona Zoo were taken to the laboratory for anesthetized blood sampling and subsequently released. Blood was drawn from the wing vein with heparinized syringes and stored in tubes in crushed ice. The packed-cell volume was 40–45%. Blood was drawn from anesthetized sparrows and Sprague–Dawley rats by cardiac puncture. The sparrows had been captured using Japanese nets.

Red blood cells were treated with 1.8 M trifluoroacetic acid to extract the inositol phosphates and related substances, and to denature proteins for further separation by centrifugation and ultramicrofiltration. The blood erythrocytes were washed three times in 0.15 M NaCl for bird cells and 0.14 M NaCl for rat and reptile cells, the leukocyte band being removed each time. Organic phosphates were obtained by two successive 30-min extractions, each using three volumes of 20% TFA for each volume of initial erythrocyte suspension. Each extract sample was kept on ice and centrifuged for 5 min at approximately 800 × g at 4 °C. The two supernatants were mixed and centrifuged for 30 min at 30 000 × g at 4 °C in an ultracentrifuge (XL-80 Beckman, USA) to precipitate the remaining proteins. The supernatants were distributed in 1.5-ml microcentrifuge tubes and evaporated in a rotary evaporator (SpeedVac SC110, Savant, USA) to concentrate the sample and remove the TFA. Finally, evaporated samples were resuspended with a low volume of deionized water and subjected to centrifugal filtration (Ultrafree-MC 10000 NMWL, Millipore, USA).

The chromatographic analysis was based on the method described by Mayr (1988), incorporating the modifications described by Casals et al. (2002). Briefly, the inositol polyphosphates from 100 µl of processed samples were separated using a Resource-Q column (Amersham-Pharmacia, Uppsala, Sweden). An increasing gradient of hydrochloric acid was applied. The chromatographic pump (Pharmacia-LKB 2150, Amersham-Pharmacia, Uppsala, Sweden) was set at flow rate of 2.75 ml/min. For samples with a very low
Fig. 1. Determination of minor inositol phosphates in pigeon, cock, and sparrow erythrocytes. HPLC analysis was performed on TFA erythrocyte extracts as described in Section 2. The buffer/PAR flow was 2.75:1.2 ml/min. The amount of erythrocytes applied was approximately 225 μl for pigeon, 220 μl for cock and 120 μl for sparrow. Inositol phosphates were identified by their elution time.

3. Results

The results of the chromatographic analysis of erythrocyte inositol phosphates for pigeon, cock and sparrow are shown in Fig. 1, while those for the three crocodilian species studied are shown in Fig. 2. The latter also includes the rat sample to provide an example of mammal erythrocytes. Minor inositol phosphates detected in the birds were the tetraphosphates Ins(3,4,5,6)P₄ and Ins(1,3,4,6)P₄, which are thought to be the main precursors of Ins(1,3,4,5,6)P₅. These isomers could not be detected in the crocodilian erythrocytes, perhaps because of the very low levels of inositol phosphates in this group. In addition to the major inositol phosphate isomer present in bird erythrocytes [Ins(1,3,4,5,6)P₅], the erythrocytes of the three bird species contained the most phosphorylated inositol form (InsP₆).

The red blood cells of the three crocodilian species contained low levels of nucleotides ATP and GTP, and some BPG. This was particularly evident in the case of Caiman crocodilus due to the larger sample and the more sensitive method applied. However, the most notable feature was the presence of highly phosphorylated inositols, Ins(1,3,4,5,6)P₅ and InsP₆, which have not previously been detected in studies using liquid chromatography analysis.

The erythrocyte concentrations of separated inositol phosphates from the crocodilian and avian species studied are shown in Table 1. Information
Fig. 2. HPLC analysis of erythrocyte organic phosphates from three crocodile species and rat. Chromatograms were developed as described in Section 2. The buffer/PAR flow was 2.75:1.2 ml/min in *Caiman latirostris* and *Osteolaemus tetraspis*, and 1:0.4 ml/min in *Caiman crocodylus* and *Rattus norvegicus*. The amount of erythrocytes applied after TFA extraction was approximately 150, 220, 480 and 160 µl, respectively. Peaks were identified according to elution time.

Table 1

Levels of inositol polyphosphates in bird and crocodile erythrocytes

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentration (µmol/ml erythrocytes)</th>
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<tbody>
<tr>
<td></td>
<td>Ins(1,3,4,6)P₄</td>
</tr>
<tr>
<td>Pigeon <em>Columba livia</em></td>
<td>7–9</td>
</tr>
<tr>
<td>Cock <em>Gallus domesticus</em></td>
<td>4–5</td>
</tr>
<tr>
<td>Sparrow <em>Passer domesticus</em></td>
<td>4–5</td>
</tr>
<tr>
<td><em>Osteolaemus tetraspis</em></td>
<td>4</td>
</tr>
<tr>
<td><em>Caiman latirostris</em></td>
<td>2</td>
</tr>
<tr>
<td><em>Caiman crocodylus</em></td>
<td>2</td>
</tr>
</tbody>
</table>

Values obtained from the chromatographic system and metal–dye detection as described in the text. Values are mean±S.D.; ND, not detected.
is biased towards synthesis. In any case, the levels of this inositol phosphate are at least two-fold higher than those of the tetraphosphate forms detected in the birds studied here. In the case of the cock, the hexaphosphate form was present in even greater quantities.

4. Discussion

4.1. Analysis of the InsP₄ isomers found in bird erythrocytes

In pigeon, cock and sparrow erythrocytes, the main InsP₄ isomer elutes at the same time as the standard Ins(3,4,5,6)P₄. However, in turkey erythrocytes the main InsP₄ isomer has been identified as Ins(1,4,5,6)P₄ (Radenberg et al., 1989). As both isomers elute at the same chromatographic time (Guse and Emmrich, 1992), it is not possible to establish which form is actually present in the erythrocytes. The isomer Ins(3,4,5,6)P₄ has been identified as the main precursor of Ins(1,3,4,5,6)P₄ in turkey erythrocytes (Stephens and Downes, 1990), which suggests that it is indeed Ins(3,4,5,6)P₄, produced by the specific phosphorylation of Ins(3,4,6)P₃ (Stephens and Downes, 1990), that is present in the pigeon, cock and sparrow erythrocytes. Moreover, the isomer Ins(1,3,4,5,6)P₄, which was also found in these birds, could be synthesized by specific phosphoinositol 3-kinase from Ins(1,4,6)P₃, as has been suggested for turkeys (Stephens and Downes, 1990). These results seem to indicate that pathways of inositol polyphosphate synthesis in the erythrocytes of species as diverse in their flight activity as pigeon, chicken and sparrow are similar, if not identical, to those in turkey erythrocytes. This is consistent with the hypothesis that they may have evolved from a common group that was the predecessor to birds.

4.2. Analysis of highly phosphorylated inositols in crocodilian erythrocytes

The only phosphates previously detected in crocodilian erythrocytes were ATP and BPG when enzymatic techniques were used (Grigg et al., 1993), and ATP and GTP (though not BPG) using chromatographic techniques (Bartlett, 1980). Although these molecules were not quantified due to the fact that the analyses could not be carried out in conditions that avoided hydrolysis, as a point of comparison we could indicate that data obtained allowed us to estimate that the order of magnitude of ATP concentration was at least 10-fold higher than InsP₄.

The InsP₅ and InsP₆ erythrocyte levels reported in the three crocodilian species in the present study are plainly higher than those in rat—5 to 10 times higher than in Jurkat lymphocytes (calculated from previously published data by Guse and Emmrich, 1992, assuming a Jurkat cell volume of $1.35 \times 10^3 \mu^3$/cell)—and slightly higher than the results obtained here for the mouse macrophages. This indicates that their level is markedly higher than might be expected, were we to consider their purely metabolic or transductional requirements. Having said this, the phosphate/hemoglobin ratio implied by these concentrations of InsP₅ and InsP₆ would be too low (assuming an erythrocyte hemoglobin concentration of 4 mM, the molar ratio for InsP₅ and InsP₆ together would be approximately 1/180) to induce changes in the hemoglobin oxygen affinity. In addition, adult crocodilian hemoglobin has a very low intrinsic sensitivity to organic phosphates, since only two of the six β-chain sites that interact with the negative charges of organic phosphates in bird hemoglobin (β1Val, β2Hys, β82Lys, β135Arg, β139Hys and β143Arg in chicken) are occupied by positively charged amino acids (β135Arg and β82Arg in Crocodylus niloticus). However, both embryonic (Grigg et al., 1993) and adult (Weber and White, 1994) crocodilian hemoglobins have some sensitivity to BPG and especially to ATP, possibly because they are smaller and fit better at the hemoglobin-binding site than InsP₆ (Weber and White, 1994). It is interesting to note that hypoxia reduces the erythrocyte concentration of ATP and GTP in rainbow trout (Val et al., 1995) and many other fish (Nikinmaa, 2001), playing an adaptive role in blood oxygen transport. InsP₅ is a much less alterable molecule, at least in birds (Riera et al., 1991). In crocodilian erythrocytes, both possibilities are open, i.e. regulation of affinity through changeable nucleotide concentrations or through inositol. The function of these inositols, therefore, cannot be related, at present, to oxygen transport.

We believe that crocodilian erythrocytes should be viewed as metabolically ‘preadapted’ to increase their InsP levels more readily, and thereby interact with the hemoglobin. This process would imply that the function of inositol phosphates has shifted from that of intracellular signaling to one
of a hemoglobin allosteric effector. Although immature erythrocytes have active inositol-dependent transduction pathways (Wasilenko, 1992; Ren et al., 1994) that might be activated if changes in the inositol levels were taking place, in fact, during the late maturation stage transduction becomes negligible and thus the concentration of inositol polyphosphates can increase without functional risk. It has been shown in anemic quail that the InsP₃ level increases during the later phases of erythrocyte maturation (Riera et al., 1991) and that adult bird erythrocytes retain an active inositol metabolism (McPhee et al., 1991). Moreover, stimulation of mammalian cells easily activates the synthesis of highly phosphorylated inositols with no evident function (Stephens et al., 1988; Casals et al., 2002). This indicates that synthesis of inositol phosphates is biased towards highly phosphorylated forms.

The fact that bicarbonate and organic phosphates bind to different regions of the hemoglobin would seem to facilitate a hypothetical transition from one allosteric effector to another (Schweitzer and Marshall, 2001). Thus, following an increase in phosphate concentration, cells such as crocodilian erythrocytes might have developed an allosteric interaction between hemoglobin and highly phosphorylated inositols by the progressive replacement of just a few amino acids in key positions (i.e., replacement of uncharged amino acids by positively charged ones: β143Ala→Arg, β139Ala→Hys and β2Ser→Hys). ATP would have been the first phosphate to reach minimum levels that would have decreased the oxygen affinity in adult erythrocytes, after which a stronger interaction could have been obtained with inositos. This would account for the presence of both phosphates in bird erythrocytes. A positive selection in terms of an increase in both phosphate concentration and the hemoglobin–phosphate interaction would have increased the oxygen released to tissues, which would have permitted an increase in the metabolic rate.

Whatever the case, our findings with regard to InsP5 (a highly representative molecule in bird erythrocytes) and InsP6 in crocodiles would seem to strengthen claims as to the similarity of the two groups and give added credibly to the idea that they might have evolved from a common group (namely Archosauria), as suggested in a number of evolutionary studies.

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