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Activation of a $P2Y_4$-like purinoceptor triggers an increase in cytosolic $[Ca^{2+}]$ in the red blood cells of the lizard *Ameiva ameiva* (Squamata, Teiidae)

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Abstract

An increasing number of pathophysiological roles for purinoceptors are emerging, some of which have therapeutic potential. Erythrocytes are an important source of purines, which can be released under physiological and physiopathological conditions, acting on purinergic receptors associated with the same cell or with neighboring cells. Few studies have been conducted on lizards, and have been limited to ATP agonist itself. We have previously shown that the red blood cells (RBCs) of the lizard *Ameiva ameiva* store Ca^{2+} in the endoplasmic reticulum (ER) and that the purinergic agonist ATP triggers a rapid and transient increase of $[Ca^{2+}]_c$ by mobilization of the cation from internal stores. We also reported the ability of the second messenger IP_3 to discharge the ER calcium pool of the ER. Here we characterize the purinoceptor present in the cytoplasmic membrane of the RBCs of the lizard *Ameiva ameiva* by the selective use of ATP analogues and pyrimidine nucleotides. The nucleotides UTP, UDP, GTP, and ATPγS triggered a dose-dependent response, while interestingly 2MeSATP, 2ClATP, α,ß-ATP, and ADP failed to do so in a 1- to 200-µm concentration. The EC_{50} obtained for the compounds tested was 41.77 µM for UTP, 48.11 µM for GTP, 53.11 µM for UDP, and 30.78 µM for ATPγS. The present data indicate that the receptor within the RBCs of *Ameiva ameiva* is a $P2Y_4$-like receptor due to its pharmacological similarity to the mammalian $P2Y_4$ receptor.

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- UTP

Introduction

Calcium regulates a myriad of physiological processes in organisms ranging from protozoa (1-5) to vertebrates (6,7) by an orchestrated cytosolic elevation and/or calcium influx (8). Since the early recognition of the potent extracellular actions of ATP and adenosine by Drury and Szent-Györgyi, in 1929 (9), considerable knowledge has been accumulated about the receptors involved in transducing nucleotide signals (10,11).

Extracellular purines (adenosine, ADP, ATP) as well as pyrimidines (UDP and UTP) play an important role in signaling events, mediating many biological effects through

receptors on the cell surface, including smooth muscle contraction, neurotransmission, exocrine and endocrine secretion, the immune response, inflammation, plaque aggregation, pain, and modulation of cardiac function. These receptors are named purinergic receptors (10).

Purinergic receptors are divided into two major families, P1 or adenosine receptors, and P2, which recognize ATP, UTP and UDP. P2 receptors are subdivided into P2Y, the G protein coupled receptors, and P2X, coupled to ionic channels. In mammals, 8 subtypes of P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$) (12) and, recently, $P2Y_{12}$ (13), $P2Y_{13}$ (14) and $P2Y_{14}$ (15) have been identified thus far.

Metabotropic $P2Y_{1-14}$ receptors are characterized by a subunit topology involving an extracellular N terminus and intracellular C terminus, the latter possessing consensus binding motifs for protein kinases, and seven transmembrane-spanning regions, which help to form the ligand-docking pocket. Each P2Y receptor binds to a single heterotrimeric G protein (typically $G_{q/11}$) although $P2Y_{11}$ can couple both with $G_{q/11}$ and G_s, whereas $P2Y_{12}$ and $P2Y_{13}$ couple with G_i and $P2Y_{14}$ couples with $G_{i/0}$ (16).

The recently cloned P2Y receptors $P2Y_{13}$ are present mainly in spleen, brain, lymph nodes, and bone marrow, whereas $P2Y_{14}$ receptors are present in placenta, adipose tissue, stomach, intestine, and discrete brain regions (17).

An increasing number of pathophysiological roles for purinoceptors are emerging, some of which have therapeutic potential (18-21). Bladder incontinence, contraception and fertility, skin diseases, diabetes, thrombosis, gut motility disorders, cardiopulmonary diseases, cancer, diseases of the ear, diseases of the eye, behavioral disorders, bone disorders, and pain (19,20) are related in one way or other to responses to purinergic receptors.

Most of the studies concerning the functional distribution of purinoceptors were carried out on mammalian preparations even though purinoceptors are widespread throughout the evolutionary scale. Evidence of their presence is found even in protozoa, where ATP was found to have an inhibitory effect on ameboid movement (10).

Few studies have been conducted on lizards, and have been limited to ATP agonist itself: an excitatory innervation has been found in the ileum of the lizard *Tiliqua rugosa* (11) and in the rectum and portal vein of the lizard *Agama agama* (22,23). In all cases, the subtype of the receptors involved was unknown.

Erythrocytes are an important source of purines, which can be released in physiological and physiopathological conditions, acting on purinergic receptors associated with the same cell or with neighboring cells (24).

Red blood cells (RBCs) of the lizard *Ameiva ameiva* loaded with the fluorescent Ca^{2+} indicator Fluo-3 AM store Ca^{2+} in the endoplasmic reticulum and in one or more acidic pools endowed with an H^+ pump sensitive to the inhibitors 7-chloro-4-nitrobenz-2-oxa-1,3-diazole and bafilomycin. Moreover, the internal Ca^{2+} pools of the RBCs of the lizard are sensitive to surface receptor stimulation since the purinergic agonist ATP stimulates the release of Ca^{2+} in a process that is hardly affected by the removal of external Ca^{2+} but is inhibited by suramin and PPADS (pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate), non-specific purinergic inhibitors (25,26). These data strongly suggest the presence of a G-coupled purinergic receptor in RBCs of the lizard *Ameiva ameiva*.

The objective of the present study was to investigate the subtype of this receptor by challenging the RBCs of *Ameiva ameiva* with ATP analogues (ATPγS, 2-methylthioATP (2MeSATP), α,ß-ATP, and 2-chloroATP (2ClATP)), ADP, GTP, and pyrimidine nucleotides (UDP, UTP) and monitoring the concentration of calcium by spectrofluorometric measurement of Fluo-3 AM.

Material and Methods

Material

2MeSATP, 2-ClATP, ADP, ATP, UDP, UTP, and GTP were purchased from Sigma, St. Louis, MO, USA. Fluo-3 AM was purchased from Molecular Probes, Eugene, OR, USA.

Lizards

The lizards *Ameiva ameiva* were captured with Tomahawk traps in the town of Barretos (20º 33' S, 48º 30' W), State of São Paulo, Brazil, and by hand in the town of Lajeado (10º 43' S, 48º 24' W), State of Tocantins, Brazil. The blood, collected from the lizard's tail with a syringe, was centrifuged at 1500 *g* for 5 min and washed in phosphate-buffered isotonic saline (7.5 mM sodium phosphate and 137 mM NaCl, pH 7.2). Leukocytes were removed from RBCs by blood filtration through a cellulose powder column (long fibers; Whatman CF11, Madstone, Kent, UK) according to the method of Homewood and Neame (27).

RBC loading with the calcium indicator Fluo-3 AM

RBCs were washed twice in buffer (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM D-glucose, and 50 mM Mops, pH 7.2) and resuspended at 10^6 cells/ml in the same buffer containing 1.8 mM probenecid, an inhibitor of organic anion transport (28), to prevent fluorochrome release and sequestration (29). Fluo-3 AM was added at a final concentration of 5 µM. The suspension was incubated for 1 h at 37ºC followed by three washes with buffer to remove the extracellular dye. In each experiment an aliquot of 100 µl (10^6 cells) was placed in a thermostated cuvette equipped with magnetic stirring. Control experiments with the solvent alone showed no measurable change in fluorescence.

Spectrofluorometric measurements with Fluo-3 AM were performed using a model F-4500 Hitachi spectrofluorometer (Tokyo, Japan) with excitation at 505 nm and emission at 530 nm. The excitation and emission slits were 1 mm.

The curves for free Ca^{2+} concentration were calculated from fluorescence measured using the Ca^{2+} software F-4500 Intracellular Cation Measurement System-Version 1.02 (Copyright© Hitachi, 1994-1995), which takes into account that $[Ca^{2+}] = K_d (F - F_{min}/(F_{max} - F)$, where the K_d utilized for Fluo-3 is 390, F is the fluorescence intensity measured under the conditions of the experiment, F_{max} the fluorescence in the presence of digitonin, and F_{min} the fluorescence in the presence of 8 mM EGTA. Unless otherwise specified, all experiments were performed at 37ºC.

The results are reported as means ± SEM (N = 3) and data were analyzed statistically by two-way ANOVA.

Results and Discussion

Figure 1 shows that the different concentrations (100 and 800 µM UTP) were able to trigger the increase of $[Ca^{2+}]_c$ in RBCs of the lizards *Ameiva ameiva* loaded with 5 µM Fluo-3 AM.

As shown in Figure 1D, with the aid of confocal microscopy we were able to observe the same effect of calcium mobilization by a purinergic agonist, in this case, 100 µM UTP. Moreover, the addition of increasing concentrations of ATPγS, GTP, UDP, and UTP (from 1 to 800 µM) led to a dose-dependent increase of $[Ca^{2+}]_c$ (data not shown).

The EC_{50} was 41.77 µM for UTP (Figure 2A), 48.11 µM for GTP (Figure 2B), 53.11 µM for UDP (Figure 2C), and 30.78 µM for ATPγS (Figure 2D).

The ATP analogues tested, 2MeSATP, α,ß-ATP, 2ClATP, as well ADP in a range of 1 to 200 µM failed to promote an increase of $[Ca^{2+}]_c$ in the RBCs (data not shown).

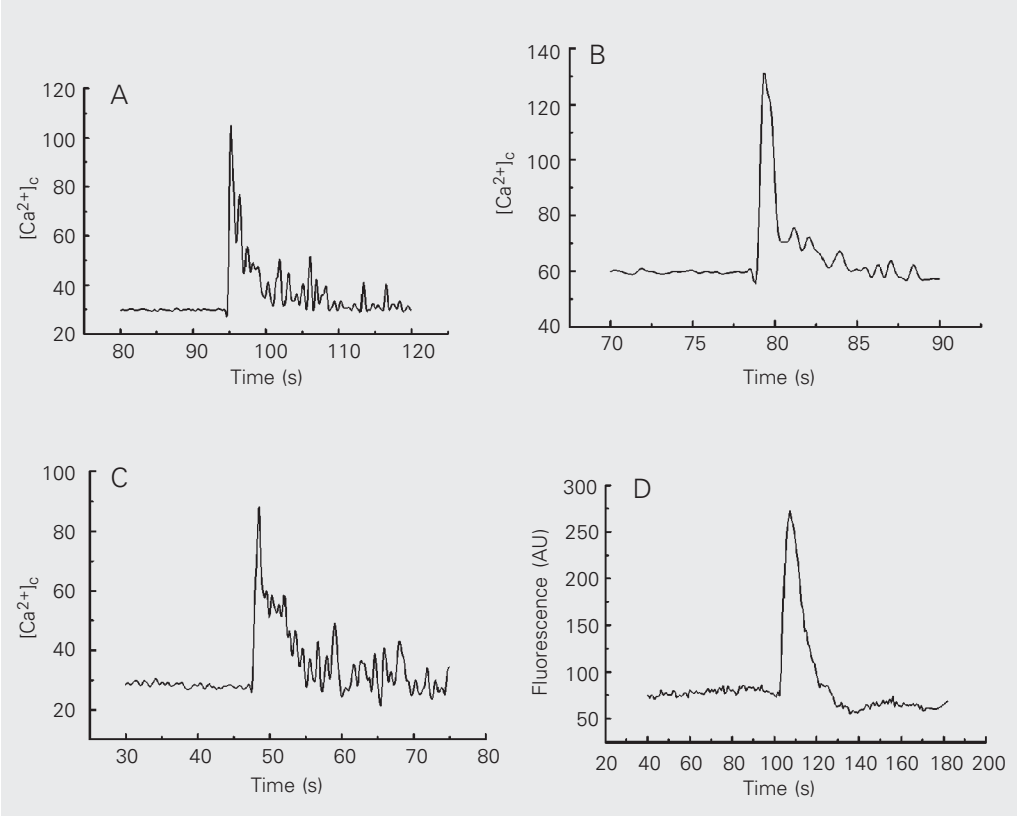

Figure 1. Effect of UTP on lizard red blood cells. Cytosolic calcium concentration (A, B, C) was measured fluorometrically with Fluo-3 AM. *A*, 100 µM UTP; *B*, 800 µM UTP in nominally calcium-free medium; *C*, 800 µM UTP in 2 mM EGTA medium; *D*, 100 µM UTP in Fluo-3 AM-loaded lizard red blood cells observed by confocal microscopy and reported in arbitrary fluorescence units (AU). The fluorescence data are qualitative. Note that the ordinate scales of *panels A*, *B* and *C* are not equal: B > A > C. Cytosolic calcium concentration is reported in nM.

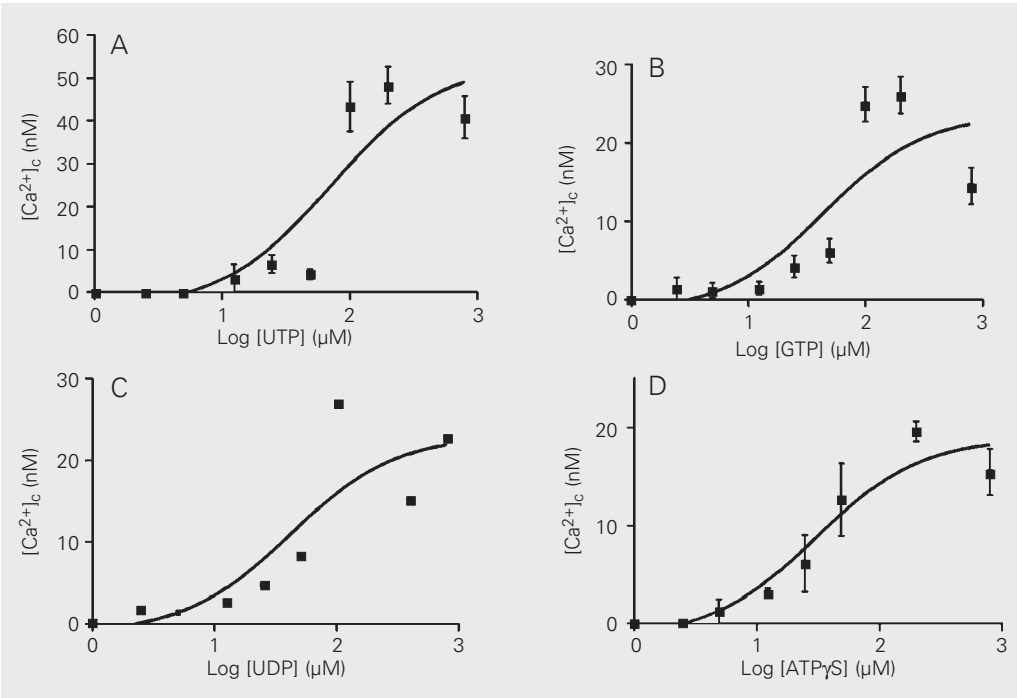

Figure 2. Dose-response curves for *A*, UTP; *B*, GTP; *C*, UDP, and *D*, ATPγS in Fluo-3 AM-loaded red blood cells of the lizard *Ameiva Ameiva*. $[Ca^{2+}]_c$ was measured by loading red blood cells with Fluo-3 AM as described in Methods. Data are reported as means ± SD for three measurements for *panels A*, *B* and *D*.

We have pharmacologically characterized the P2Y receptor in *Ameiva ameiva* lizard RBCs. For this purpose, a set of purinergic analogues were tested (Table 1). Dose-response curves were obtained for the agonists which responded: ATPγS, GTP, UDP, UTP, 2MeSATP, 2ClATP, α,ß-ATP and ADP. The subtype identification has been inferred on the basis of sensitivity to different types of agonists, the potency relationships among them and the ability to inhibit the response. The receptor under study was pharmacologically characterized according to the criteria described below.

A typical trait of P2Y receptors is that they respond differently to natural agonists such as ATP and UDP and their diphosphate and triphosphate analogues (11).

The $P2Y_1$, $P2Y_{11}$ and $P2Y_{12}$ receptors are selective for adenine nucleotides, while the others can be stimulated by uracil nucleotides. The $P2Y_1$ and $P2Y_{12}$ receptors are equally responsive to 2MeSATP and ATP.

The $P2Y_2$ and $P2Y_4$ receptors are equally responsive to ATP and UTP, and are not responsive to 2MeSATP (21). The $P2Y_6$ receptor is selectively activated by UDP (15). Suramin is a non-selective P2 receptor antagonist which competitively antagonizes $P2Y_1$ and $P2Y_2$. The PPADS antagonist competitively antagonizes $P2Y_1$ and $P2Y_4$ receptors, but not $P2Y_2$ (30).

The $P2Y_{12}$ receptor, recently cloned from human platelets, is stimulated by ADP and ATP and selectively blocked by 2MeSATP (31).

Since addition of the uracil nucleotides UTP and UDP to lizard RBCs elicits a Ca^{2+} response, the presence of the receptors $P2Y_1$, $P2Y_{11}$ and $P2Y_{12}$ can be eliminated. Furthermore, since similar to adenine nucleotides (ATP), UDP also promotes a Ca^{2+} response in these cells, the $P2Y_6$ receptor subtype can also be ruled out. Since PPADS were able to antagonize the response promoted by ATP addition, we excluded the participation of $P2Y_2$ receptor signaling in these cells. Finally, $P2Y_4$ seems to be the receptor involved in transducing the signal within the RBCs of *Ameiva ameiva* lizards.

Table 1. Calcium response of red blood cells of the lizard *Ameiva Ameiva* to nucleotide agonists.

Nucleotide	Ca^{2+} response
ATPγS	↑
GTP	↑
UDP	↑
UTP	↑
2MeSATP	NR
2ClATP	NR
α,ß-ATP	NR
ADP	NR

The experimental conditions are described in Methods. Typical concentrations used are reported for positive responses (arrows) and maximum concentrations are reported for no response (NR).

However, all of these considerations are based on mammalian models, and the denomination $P2Y_4$-like receptor seems to be more appropriate to designate the receptor found on the surface of *Ameiva ameiva* RBCs until structural information is available.

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