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Activation of calcium-insensitive phospholipase A_2 ($iPLA_2$) by $P2X_7$ receptors in murine peritoneal macrophages

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ABSTRACT

Free fatty acid releases are triggered by PLA2 activation and are substrates for many enzymes such as cyclooxygenases. These reactions are responsible for the production of many prostaglandins implicated in the inflammation yet many purinergic receptors have been implicated in diseases characterised by chronic inflammation. The role of P2X receptors was evaluated in LPS-primed murine peritoneal macrophages which were labelled with either [^{3}H]-oleic acid or [^{3}H]-arachidonic acid. Ten μmolar thapsigargin and 1 mM ATP stimulated the release of both unsaturated acids. ATP had no effect at 10 μM and ivermectin had no effect on the response to ATP. The response to ATP was inhibited by magnesium and was not observed with cells from $P2X_7^{-/-}$ mice. The response to ATP was not affected by the removal of extracellular calcium and was inhibited by arachidonyltrifluoromethyl ketone and bromoenol lactone but not by pyrrophenone. The release of the [^{3}H]-fatty acids by ATP and thapsigargin was diminished by PD-98058, an inhibitor of MEK-1. It was concluded that in LPS-primed macrophages, $P2X_7$ receptors, not $P2X_4$ receptors, activated an $iPLA_2$ and promoted the release of unsaturated fatty acids secondary to the activation of a kinase. This response might contribute to the inflammation provoked by extracellular ATP.

1. Introduction

Purinergic extracellular nucleotide receptors have been classified into 2 major groups. P2Y receptors are metabotropic receptors which have 7 transmembrane domains and which are coupled to a transducing unit, a GTP-binding protein. P2X receptors are ionotropic receptors; they have only 2 transmembrane domains and after ligation of an agonist, they form trimeric structures which transfer positive electrical charges through the plasma membrane. Seven P2X subunits have been cloned. They average 400–450 amino acids and possess short intracellular N- and C-terminal domains. The only exception is the $P2X_7$ receptor which is longer than the 6 other P2X receptors (nearly 600 amino acids) [1]. $P2X_7$ receptors are, among all the P2 receptors, the receptors with the lowest affinity for ATP [2]. The presence of magnesium inhibits the responses mediated by the $P2X_7$ receptor by decreasing the extracellular concentration of ATP^{4-}, the natural agonist of all P2X receptors [3]. This receptor has a very long C-terminal domain which interacts with several intracellular proteins and which confers unique properties to this receptor [4]. It does not desensitise and after prolonged stimulation with a high concentration of ATP, it forms a pore permeant to large molecules [5]. This receptor was initially described in immunocytes [3]. In macrophages, the $P2X_7$ receptors have been coupled with the activation of caspase-1 and the secretion of IL-1β [6], the killing of intracellular pathogens [7], phagocytosis and efferocytosis [8], apoptosis [9], the production of reactive oxygen species (ROS) [10], the formation of multinucleated giant cells [11] and membrane blebbing [12]. The $P2X_7$ receptors trigger these multiple responses by forming, in the plasma membrane, a channel selective to cations and which increases the intracellular concentration of calcium [13], decreases the intracellular concentration of potassium [14] and depolarises the plasma membrane [2]. The formation of a pore by the receptor [3] or the interaction of its intracellular C-terminal domain with proteins or lipids [2] also affects several pathways. The $P2X_7$ receptors regulate the activity of various protein kinases and phosphatases like protein kinase C [15], protein kinase D [16], mitogen-activated protein kinase [17], tyrosine phosphatase [18], and of various lipases like phospholipase C [16], phospholipase D [19], phospholipase A_2 (PLA_2) [20] or sphingomyelinase [21].

$P2X_4$ receptors, like $P2X_7$ receptors, are expressed by immune cells and have been implicated in inflammation [22–24]. $P2X_4$ receptors, unlike other purinergic receptors have no specific antagonist and can be allosterically modulated by ivermectin [25]. We recently reported that the activation of these receptors promoted the efflux of potassium and decreased the intracellular concentration of this ion both in macrophages and in submandibular glands [26]. This, in turn, stimulated the secretion of IL-1β [24,26].

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The $P2X_4$ receptors expressed by microglial cells might contribute to allodynia [27]. The group of Rassendren reported that in resident peritoneal macrophages, the activation of $P2X_4$ receptors stimulated the activity of PLA_2 and the release of arachidonic acid (AA) and prostaglandins [28]. Phospholipases A_2 are enzymes which hydrolyse the ester link between the secondary alcohol group of glycerol and the carboxylic function of an unsaturated fatty acid. According to these authors, the activation of PLA_2 in macrophages located in the central nervous system might play a major role in pain [28]. The purpose of our work was to reconsider the regulation of PLA_2 by ATP in murine macrophages. We specially examined the identity of the enzyme regulated by the nucleotide and the classes of purinergic P2X receptors involved in this regulation. Macrophages express two cytosolic phospholipases A_2, the calcium-dependent ($cPLA_2$) and the calcium-insensitive PLA_2 ($iPLA_2$) [29,30]. Determination of the identity of the PLA_2 implicated in free fatty acid release was carried out by the use of radiolabelled fatty acids (oleic and arachidonic acids), a calcium chelator (ethylene glycol tetraacetic acid (EGTA)) and several inhibitors: bromoenol lactone (BEL), arachidonyltrifluoromethyl ketone (AACOCF3) and pyrrophenone. AACOCF3 is an analog of AA which slowly but tightly inhibits the calcium-dependent $cPLA_2$ [31,33], BEL is an inhibitor of the calcium-insensitive PLA_2 ($iPLA_2$) [32] and pyrrophenone is a specific $cPLA_2$ inhibitor [33].

2. Materials and methods

2.1. Materials

Ethylene glycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid] (HEPES), adenosine 5′-triphosphate disodium salt (ATP), fetal bovine serum (FBS), lipopolysacharides from *Escherichia coli* (LPS), ivermectin, thapsigargin, bromoenol lactone, β-NADH and sodium pyruvate were obtained from Sigma–Aldrich (St. Louis, MO). The glutamine-free amino acids mixture, the medium RPMI-1640, L-glutamine, the phosphate-buffered saline solution (PBS) and the solution of penicillin–streptomycin were from GIBCO (Invitrogen, Paisley, Scotland). 9,10-[^{3}H]-oleic acid ([^{3}H]-OA) and 5,6,8,9,11,12,14,15-[^{3}H]-arachidonic acid ([^{3}H]-AA) were purchased from PerkinElmer. (Zaventem, Belgium). The scintillation solution Ecoscint A was from National Diagnostics (Atlanta, GA). Thioglycollate was supplied by Becton-Dickinson (Franklin Lanes, NJ). Bovine serum albumin (BSA) was provided by Roche Diagnostics (Vilvoorde, Belgium). PD-98059, arachidonyltrifluoromethyl ketone and RO 32-0432 were from Calbiochem (La Jolla, CA). Pyrrophenone was from Bio-Connect (TE Huissen, The Netherlands). Fluo-4 AM was from Invitrogen (Groningen, The Netherlands).

The experiments were carried out on C57Bl/6J $P2X_7R^{+/+}$ wild-type mice and $P2X_7R^{-/-}$ mice kindly supplied by Pfizer Inc. (Groton, CT) [5]. Breeding of male with female $P2X_7R^{-/-}$ mice was used to maintain the colony of receptor-deficient animals. Mice used in the experiments were between 20–25 g. The expression of $P2X_7$ receptors in wild-type mice and its absence in null mice has previously been tested by Western blot [23].

2.2. Cell culture

Peritoneal macrophages were prepared according to McCarron et al. [34]. Briefly, peritoneal exsudates were induced by injection of 1.5 ml thioglycollate solution (4% in sterile water) 3 or 4 days before harvesting the cells. The animals were fasted overnight and sacrificed by raising the CO_2 concentration in accordance with the procedures of the Belgian Ministry of Agriculture under the supervision of the institutional ethical committee (protocol no. 221N). Ten ml sterile phosphate-buffered saline (pH 7.3) without calcium or magnesium (PBS) and containing 10 U/ml heparin were injected in the peritoneal cavity. The abdomen was gently massaged after its distension. The fluid was recovered and transferred to a sterile tube kept on ice. After centrifugation at 1500 × *g* for 10 min at 4 °C, the cellular pellet was resuspended in RPMI 1640 medium supplemented with 20 mM HEPES, 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (RPMI medium). The cells were transferred to culture flasks (usually 2 flasks per mouse) and incubated at 37 °C for at least 2 h in a humidified atmosphere containing 5% CO_2.

2.3. Assay for [^{3}H] fatty acid release

After 2 h at 37 °C, the medium was removed and the cells were incubated overnight in RPMI medium containing 250 ng/ml LPS and 1.5 μCi/ml [^{3}H]-OA or 1 μCi/ml [^{3}H]-AA. The medium was removed and the cells were washed twice with 10 ml PBS. They were then incubated for 1 h at 37 °C in fresh RPMI medium to remove the tritiated free fatty acid which had not been incorporated into phospholipids. After 2 washes with PBS, the adherent macrophages were scraped, suspended in PBS and centrifuged at 1500 × *g* for 10 min at 4 °C. The cells were then put into suspension and incubated in 500 μl HEPES-buffered saline (HBS) medium containing (mM): 24.5 HEPES (pH 7.4), 96 NaCl, 6 KCl, 2.5 NaH_2PO_4, 11.5 glucose, 5 sodium pyruvate, 5 sodium glutamate, 5 sodium fumarate, 1 $CaCl_2$, 1% (v:v) glutamine-free amino acids mixture, 0.1% (w:v) bovine serum albumin (BSA) and the tested agent for 20 min at 37 °C. Each assay was performed in triplicate. At the end of the incubation, the suspensions of 500 μl were centrifuged for 5 min at 10,000 × *g* to remove detached cells and 400 μl were transferred to scintillation vials. The radioactivity was estimated in a TriCarb liquid scintillation counter (Perkin Elmer, Waltham, MA) after the addition of 5 ml Ecoscint A. Blank and total values were estimated each time. Totals were obtained by measuring the radioactivity in whole cell suspension whereas blank values were estimated by taking 400 μl of the supernatants of cells which were centrifuged straight after their suspension in 500 μl HBS. In order to estimate the efficiency of the centrifugal step routinely used to remove all cell debris prior to [^{3}H]-AA detection, the cells were treated as described above but were centrifuged at 100,000 × *g* for 1 h at 4 °C before transferring the supernatants to scintillation vials.

2.4. Intracellular calcium increase

After an overnight incubation of peritoneal mice macrophages with or without LPS (250 ng/ml), the culture plates were washed once with PBS. Scraped cells were then incubated at 37 °C for 30 min in PBS with 2.5 μM Fluo-4 AM. At the end of this incubation, the cells were washed and diluted with HBS supplemented with 1 mM $CaCl_2$. They were transferred in the cuvette of a fluorimeter and maintained at 37 °C under constant stirring. The light emitted at 520 nm was measured every second after excitation at 485 nm (Photon Technology International, Birmingham, NJ, U.S.A.). At the end of the incubation, the cells were exposed to 100 μM digitonin to permeabilise the cells and to equilibrate the intra- and extracellular concentrations of calcium and of Fluo-4 (maximal fluorescence of the dye). The fluorescence measured at various times was normalised to baseline and to maximal fluorescence.

2.5. Statistical analysis

The statistical significance of the results was analysed by one way ANOVA test followed by the Bonferroni's Multiple Comparison Test (GraphPAD PRISM Version 4.00, San Diego, CA, USA) if the