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Extracellular ATP in the lymphohematopoietic system: P2Z purinoceptors and membrane permeabilization

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Abstract

The effects of extracellular nucleosides and nucleotides on many organs and systems have been recognized for almost 50 years. The effects of extracellular ATP (ATP_o), UTP_o, ADP_o, and other agonists are mediated by P2 purinoceptors. One of the most dramatic effects of ATP_o is the permeabilization of plasma membranes to low molecular mass solutes of up to 900 Da. This effect is evident in several cells of the lymphohematopoietic system and is supposed to be mediated by P2Z, an ATP^{4-}-activated purinoceptor. Here, we review some basic information concerning P2 purinoceptors and focus our attention on P2Z-associated phenomena displayed by macrophages. Using fluorescent dye uptake, measurement of free intracellular Ca^{2+} concentration and electrophysiological recordings, we elucidate some of the events that follow the application of ATP to the extracellular surface of macrophages. We propose a regulatory mechanism for the P2Z-associated permeabilization pore. The presence of P2 purinoceptors in cells of the lymphohematopoietic system makes them potential candidates to mediate immunoregulatory events.

Key words
- ATP
- P2Z purinoceptor
- Macrophage
- Monocyte
- T cell
- Permeabilization

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Effects of extracellular ATP

The effects of extracellular nucleosides and nucleotides on many organs and systems have been recognized for almost 50 years (1-3). Early investigators concentrated on the actions of extracellular ATP (ATP_o) and adenosine on the cardiovascular system, including their shock-inducing properties and their applications to geriatric patients with cardiovascular disorders.

In non-lymphoid tissues, both norepinephrine and acetylcholine can be found co-localized with ATP_o. Actually, in the nervous system, ATP is stored and released with norepinephrine and acetylcholine, and can act either as a neurotransmitter or as a co-transmitter (1-3). In the endocrine system, ATP_o can act as a secretagogue for hormones, as demonstrated in the pancreas and adrenals.

In the lymphohematopoietic system, one of the most dramatic effects of ATP_o is the permeabilization of plasma membranes of several cell types to low molecular mass solutes of up to 900 Da. This phenomenon requires mM concentrations of ATP_o and has already been described in macrophages, mast cells, phagocytic cells of the thymic reticulum, bone marrow cells, and thymic as

well as peripheral blood lymphocytes (3-9). At lower doses, however, ATP_o has been associated with many different phenomena. In thymocytes, ATP_o can modulate DNA synthesis, blastogenesis, and apoptosis (3,10) and in the monocytic lineage it can stimulate the synthesis of prostaglandin and leukotriene C4, induce the maturation of IL-1ß, increase the mRNA levels of nitric oxide synthase, and induce the production of reactive oxygen radicals (11-15). These data, which clearly indicate the importance of nucleotides and nucleosides as intercellular mediators, stimulated us to investigate their role as immune regulators. In the following sections we will review some of the basic properties of the P2 purinoceptors and then we will focus our attention on the P2Z purinoceptors associated with the phenomenon of macrophage permeabilization.

P2 purinoceptors

Purinoceptors mediate most of the effects of extracellular nucleotides and nucleosides. Two classes of purinoceptors, with several subtypes, have been proposed based on pharmacological, biochemical and functional data (1). P1 purinoceptors were subdivided according to the relative potencies of a series of adenine analogues and their effects on the levels of adenylate cyclase activity; P2 purinoceptors have been originally postulated on the basis of relative potencies of ATP, ADP and analogues, as well as on the basis of selective antagonisms. Based on these criteria, at least 4 P1 and 6 P2 subtypes of purinoceptors have been identified (1,3,16). P2Y and P2U purinoceptors act via the intracellular G protein cascade. P2U purinoceptors have a stronger specificity for UTP over ATP. P2T, P2X, and P2Z purinoceptors appear to act as ligand-gated channels. All P2 purinoceptors induce the increase of free intracellular Ca^{2+} concentration. Detailed pharmacological and molecular studies of P2 purinoceptors have been delayed by the lack of specific agonists and antagonists.

Several P2 purinoceptors have been recently cloned, leading to a new proposal for their classification based mainly on their homology and signal transduction mechanism (16). P2X purinoceptors ($P2X_1$-$P2X_7$) are ligand-gated ion channels that are permeable to Na^+, K^+ and, exceptionally, to Ca^{2+}, while P2Y purinoceptors ($P2Y_1$-$P2Y_6$) are G protein-coupled, leading in some cases to the mobilization of intracellular Ca^{2+}. Unfortunately, a clear association between the gene product and the physiologically expressed receptors has not yet been established for all cloned molecules.

Some of the P2 purinoceptors have been characterized in cells of the lymphohematopoietic system and other cells and tissues of interest to the immunologist (3). In macrophages, there have been reports of the expression of P2Z, P2Y, and P2U purinoceptors (3,17-19).

P2Z purinoceptors in the lymphohematopoietic system

P2Z purinoceptors are expressed in several cells of the lymphohematopoietic system, including macrophages, microglial cells, mast cells, neutrophils, megakaryocytes, multipotent hematopoietic stem cells, thymocytes, and some peripheral lymphocyte subsets (1,3,7). This receptor has also been described in some transformed cell lines and in rat parotid acinar cells (3,20). The effects of ATP_o are mediated by ATP^{4-} ions and ATP-γ-S, but not by ADP, AMP, or adenosine (1,3). Addition of Mg^{2+} inhibits the P2Z-mediated phenomenon, supposedly due to the lack of agonist action of $Mg\text{-}ATP^{2-}$ molecules.

The presence of P2Z purinoceptors can be ascertained by the activation of cation selective conductance and Ca^{2+} mobilization, together with their most striking characteristic, i.e., the permeabilization of cell membranes to low molecular mass solutes with M_r of up to 900 (3,4). In peripheral blood lymphocytes, thymocytes and hematopoietic stem

cells, permeabilization seems to be limited to molecules of M_r below 400 (3). This difference in molecular mass cut-off may reflect the existence of subtypes of the P2Z purinoceptors or a differential modulation of membrane permeability according to the cell type.

Important differences can be observed among the several manifestations of P2Z activation. Cation currents and an increase of free intracellular Ca^{2+} concentration can be detected a few milliseconds after stimulation and can be triggered at µM concentrations of ATP_o. On the other hand, membrane permeabilization is a much slower phenomenon that will only become evident several seconds or even minutes after stimulation and requires mM concentrations of ATP_o, with a maximum at 5-10 mM.

The molecular nature of P2Z purinoceptors is not fully understood and evidence exists indicating that fast-activating transmembrane cation currents and membrane permeabilization (pore formation) are indeed separate phenomena (21,22). The $P2X_7$ molecule shares several pharmacological and electrophysiological properties with P2Z purinoceptors such as the activation by ATP^{4-} and BzATP, and the induction of fast-activating cation current, leading some groups to name this molecules $P2Z/P2X_7$.

The physiological function of P2Z purinoceptors is still an open question and due to the lack of more specific pharmacological tools, the data available should be considered with caution. A role in cell death has been proposed based mainly on the permeabilization phenomenon and on the induction of apoptosis in some cell types such as thymocytes and macrophages (23). In macrophages, P2Z activation has been associated with IL-1 maturation and release (24), formation of multinucleated giant cells (19), and the elimination of macrophages infected by intracellular parasites (25). In human lymphocytes, P2Z purinoceptors have been associated with the loss of L-selectin (26).

Permeabilization of macrophages, monocytes and T cells by ATP_o

The P2Z-associated permeabilization phenomenon can be easily observed by the uptake of fluorescent dyes such as the cytoplasm space marker Lucifer yellow, and the DNA markers ethidium bromide, propidium iodine and TO-PRO-3. Figure 1 illustrates flow cytometry analysis of thioglycollate-elicited murine intraperitoneal cells and fresh human peripheral blood mononuclear cells (PBMCs) permeabilized for 10 min in the presence of high extracellular ATP concentration (Alves-Neto JL, Coutinho-Silva R and Persechini PM, unpublished data). Murine cells, mostly macrophages, were readily permeabilized (Figure 1A) while PBMCs displayed a more complex pattern of permeabilization (Figure 1B). Three-color analysis of the PBMCs showed that monocytes ($CD14^+$ cells) had a strong permeabilization pattern, reaching an intracellular fluorescence intensity 10-fold higher than that obtained in the absence of ATP (Figure 1C). On the other hand, T cells ($CD3^+$) displayed a fluorescence increase of only 3-5-fold (Figure 1D). The analysis of other cell populations present in PBMCs is currently under way. The conclusion that the above-described permeabilization is a P2Z-associated phenomenon comes from additional experiments showing that it is not induced by extracellular UTP and that it can be blocked by high (5-10 mM) extracellular Mg^{2+} concentration (Alves-Neto JL, Coutinho-Silva R and Persechini PM, unpublished data).

Changes in intracellular Ca^{2+} concentration induced by ATP_o and UTP_o and other signal transduction pathways

The pattern of the increase of intracellular Ca^{2+} concentration induced by the interaction of a P2 purinoceptor agonist may

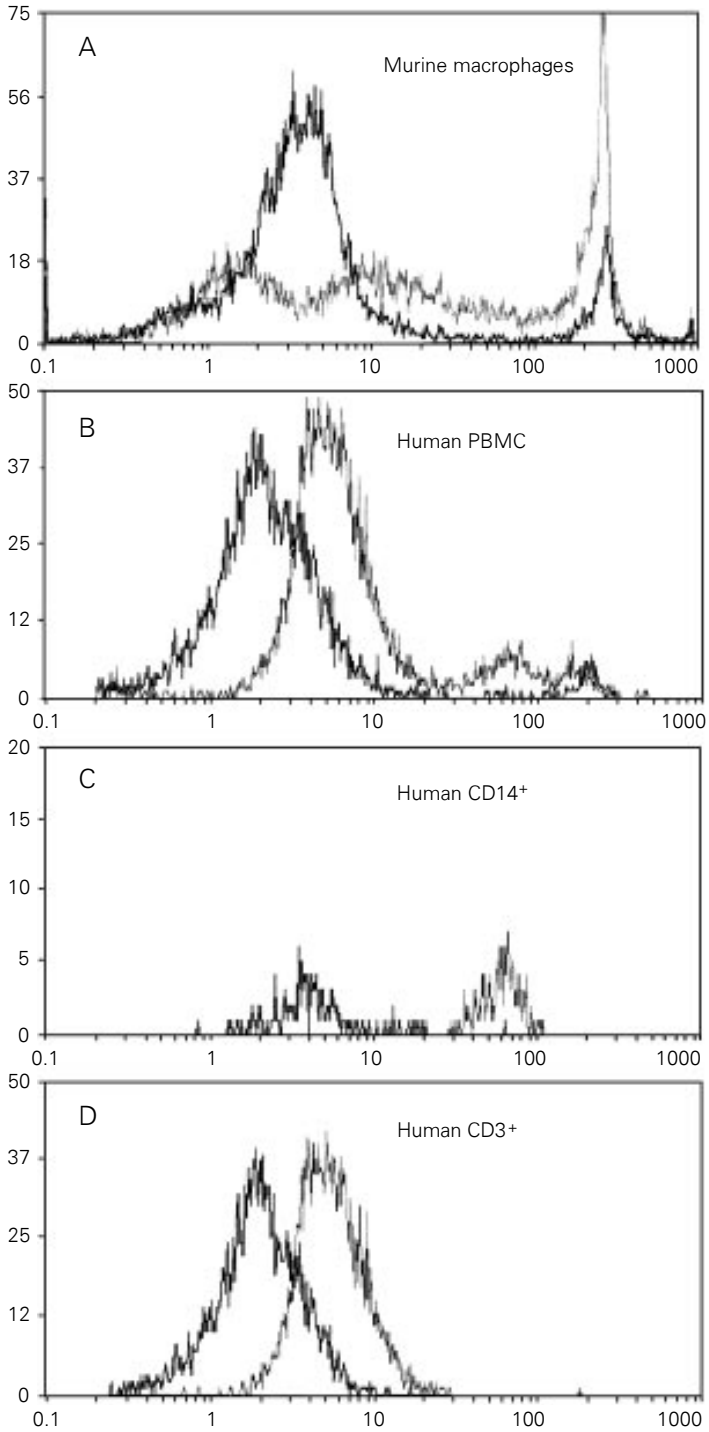

Figure 1 - ATP_o-induced P2Z-associated permeabilization of macrophages and PBMCs. Flow cytometry analysis was performed on freshly isolated thioglycollate-elicited murine intraperitoneal cells (A) and human PBMCs (B-D). Murine cells were not stained with antibodies, while PBMCs were stained with FITC-labeled anti-CD3 and PE-labeled anti-CD14 immediately before the experiment. The permeabilization assay was performed by exposing cells to either 0 mM (darker line) or 10 mM (lighter line) of extracellular ATP for 10 min at 37ºC in the presence of a fluorescent dye (ethidium bromide in A and TO-PRO-3 in B-D). Experiments were performed using a COULTER Epics-Elite flow cytometer and double excitation wavelengths: 633 nm for TO-PRO-3 and 488 nm for all other fluorochromes.

differ according to the subtype of receptors expressed by the cell and the experimental conditions. Therefore, a deeper insight into the nature of the ATP_o-induced permeabilization phenomenon can be obtained by studying cells loaded with fluorescent Ca^{2+} indicators. Figure 2 shows a representative experiment performed with adherent murine macrophages. The addition of ATP_o at 37ºC induced a fast-activating spike of Ca^{2+} followed by a slowly activating increase in intracellular Ca^{2+} concentration (Figure 2A). The concentration of Ca^{2+} may return to basal levels if ATP_o is withdrawn (Figure 2A) but a 10-20-min exposure to the agonist will usually render the process irreversible, leading to a continuous rise in intracellular Ca^{2+} concentration. This result suggests that a continuous influx of Ca^{2+} takes place as the consequence of irreversible membrane permeabilization (Figure 2B). In agreement with this hypothesis, it can be demonstrated that the slowly activating increase in intracellular Ca^{2+} concentration is also temperature dependent, becoming almost completely inhibited at 25ºC (Figure 2C). Moreover, the P2U agonist UTP, a nucleotide that does not induce membrane permeabilization, induced only a fast-activating Ca^{2+} spike both at 25 and 37ºC (Figure 2D-E).

The change in free intracellular Ca^{2+} concentration is only a part of the complete cascade of the poorly characterized signal transduction pathways employed by all P2 purinoceptors. Macrophages have P2Z, P2Y, and P2U purinoceptors (3,17,18) and, besides the receptor-gated cation channels such as the ones that will be described later in this review, candidate intermediates include G protein, phospholipases A_2 and D, and calmodulin-regulated pathways (3,22,27,28). P2Y and P2U purinoceptors may induce the release of intracellular Ca^{2+} stores, while P2Z has been shown to open G protein-coupled transmembrane Ca^{2+} channels (29) and to activate phospholipase D in macrophages (30) and lymphocytes (31). More-

over, the P2Z-associated $P2X_7$ molecule has a cytoplasmic tail that is required to induce permeabilization but not a cation current, suggesting a role in an as yet unidentified transduction pathway (32).

Ion currents, small channels and pores induced by ATP_o in macrophages

Some of the most powerful tools to study receptor pharmacology are provided by electrophysiological techniques. Plasma membranes of cells of the lymphohematopoietic system can be routinely patch-clamped, providing a high-resolution time-resolved description of many events that follow receptor-ligand interaction. Recently, a better understanding of the properties of the P2Z purinoceptor has been achieved by applying these techniques to macrophages and other cells (9,18,29,32-38).

Signal transduction mediated by all P2 purinoceptors involves the regulation of ion channels (3,27). P2Z purinoceptors are associated with membrane depolarization and permeabilization or "pore" formation, two phenomena that should induce striking modifications in membrane conductance. Early studies using macrophages and mast cells (33,39) demonstrated the existence of a non-selective cation conductance activated by ATP_o^{4-} that can induce membrane depolarization but a direct correlation between this depolarization with the phenomenon of membrane permeabilization to low molecular weight solutes has never been demonstrated. The results recently obtained in our laboratory have demonstrated the existence of a more complex pattern of transmembrane currents, allowing us to describe new unitary channels involved in these phenomena and to start to understand their regulatory mechanism (9,18,36,38).

Figure 3 illustrates whole-cell currents and unitary channels obtained by applying ATP_o to macrophages patch-clamped under

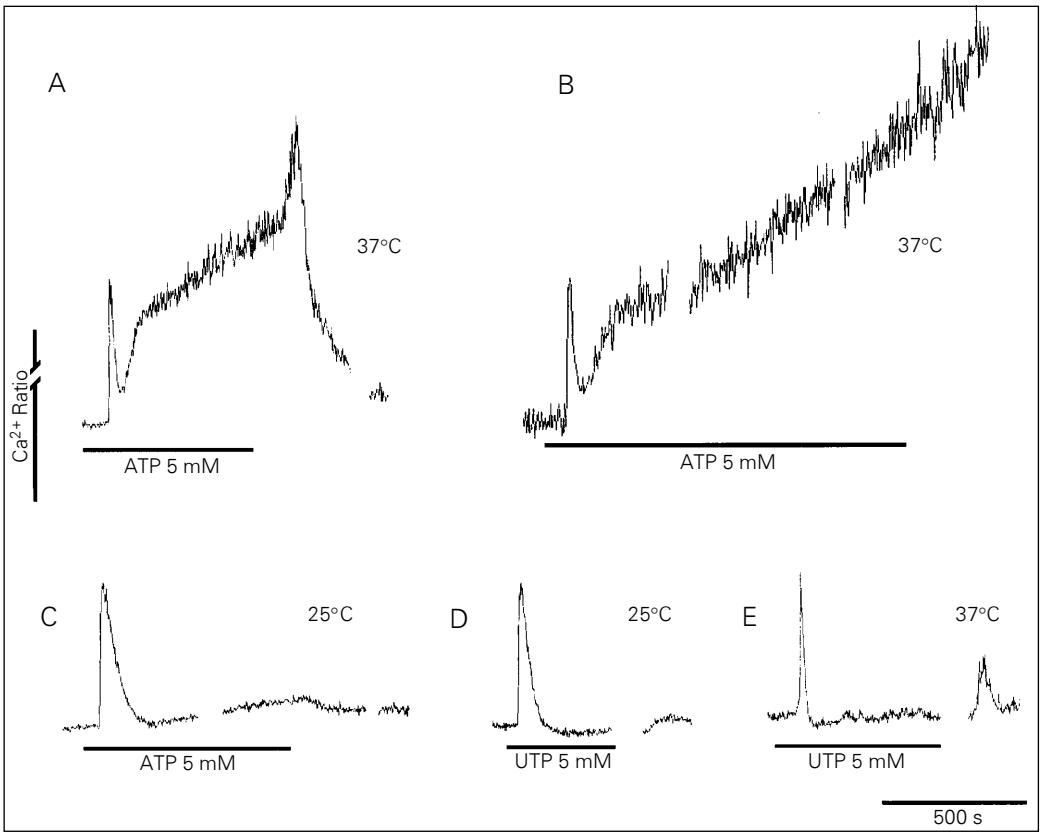

Figure 2 - ATP_o- and UTP_o-induced changes in intracellular Ca^{2+}. Thioglycollate-elicited murine macrophages were cultivated on glass coverslips for a few days and labeled with the fluorescent intracellular Ca^{2+} indicator Fura-2 AM immediately before the experiment. Fluorescence measurements were made using a microscope equipped with a spectrofluorometer that permitted the measurement of fast-activating Ca^{2+} signals of a small number of cells (30-50 cells). An extracellular solution containing 0 or 5 mM of either ATP (A-C) or UTP (D-E) was continuously perfused through the culture chamber maintaining the cells at the indicated temperature. The presence of the agonist is indicated by the horizontal bar in each record. The gaps in the records indicate irrelevant interruptions of the data storage system.

several experimental conditions. In the whole-cell configuration (Figure 3A), the application of ATP pulses close to the macrophage extracellular surface induces a biphasic transmembrane current that consists of a fast-activating inward (depolarizing) current, followed by a delayed outward (hyperpolarizing) current. Ion-exchange and other complementary experiments have demonstrated that the outward current can be ascribed to a Ca^{2+}-dependent K^+ current, while the inward current is selective for small cations such as Na^+ and K^+ (18,34,36). Therefore, the activation of macrophage P2Z purinoceptors induces two opposite phenomena: a fast-activating cation current that can depolarize the macrophage membrane, and a delayed K^+ current that drives the membrane

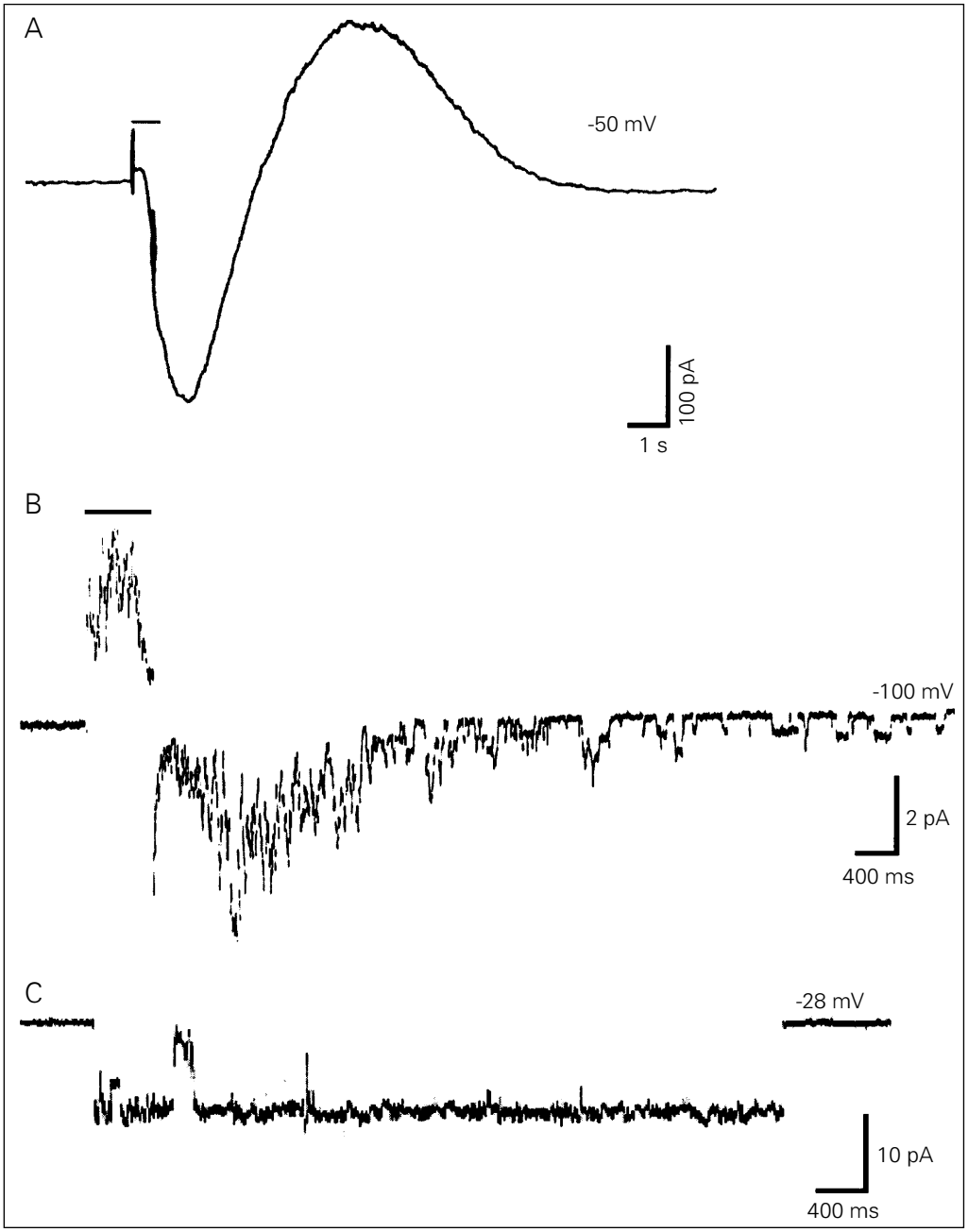

Figure 3 - Currents and ion channels associated with P2Z purinoceptors. Thioglycollate-elicited murine macrophages were placed in a heated culture chamber and patch-clamp records were obtained by a heat-polished micropipette filled with the desired solution. *A*, Whole-cell record of a macrophage; *B*, outside-out record of 8-pS single channels; *C*, cell-attached record of a pore of approximately 400 pS. In *A* and *B*, ATP was applied iontophoretically by placing the tip of a second micropipette close to the surface of the patch-clamped cell (horizontal bars on the left). In *C*, the P2Z agonist BzATP was continuously present in the extracellular medium.

potential towards more negative values.

The fast-activating depolarizing current was resolved at the single channel level using the patch-clamp technique in the outside-out configuration (Figure 3B). This type of experiment allowed us to conclude that it is a ligand-gated ion channel with a unitary conductance in the range of 5-8 pS. This channel is too small and does not support the transport of larger molecules such as Tris and NMDG and, therefore, cannot be directly involved in the transport of molecules of molecular mass up to 900 Da.

The experiments described above have elucidated a part of the cascade of events associated with signal transduction by P2Z purinoceptors. However, they have failed to describe currents and/or channels that could explain the permeabilization phenomenon. In order to solve this problem we performed patch-clamp experiments in the cell-attached configuration, a condition that allows the study of single channels without interfering with the intracellular milieu. A typical experiment is shown in Figure 3C. A few minutes after the application of ATP, a channel with a conductance of approximately 409 pS starts to open (40). These channels are voltage dependent and display several properties of the P2Z-associated permeabilization phenomenon: they are permeable to both large cations and anions, such as Tris, NMDG, and glutamate; their opening is favored at temperatures higher than 30°C; they are blocked by oxidized ATP and Mg^{2+}; they can be triggered by BzATP, but not by UTP or ADP. We thus concluded that these pores are associated with the P2Z permeabilization phenomenon and we called them "Z pores".

Experiments like the one shown in Figure 3C lead us to conclude that the opening of Z pores is regulated by second messengers since the patch of membrane that contains the pore is inside the pipette and has no access to the applied ATP.

The P2Z-associated permeabilization phenomenon is not related to connexin-43

In a separate series of experiments we also investigated the possibility that connexin-43 hemichannels, macromolecular structures most frequently associated with direct cell-cell communication, could be involved in ATP_o-induced permeabilization phenomena, the hallmark characteristic of P2Z purinoceptors. This hypothesis was supported by data indicating that connexin-43 mRNA is expressed in ATP_o-susceptible macrophages but not in ATP_o-resistant macrophages (41). We and our colleagues (37) have confirmed the presence of connexin-43 mRNA in P2Z-expressing peritoneal macrophages and J774 line; however, the ATP-induced permeabilization in macrophages was not affected either in the presence of octanol or at low pH, two conditions expected to close gap-junction pores. Moreover, ATP_o-induced Lucifer yellow uptake was observed in peritoneal macrophages derived from connexin-43 knockout mice (37). These results clearly indicate that hemigap junctions are not functionally expressed to support ATP_o-induced permeabilization.

The regulation of the P2Z-associated ATP_o-induced permeabilization: a hypothesis

The permeabilization of P2Z-expressing plasma membranes by ATP^{4-} is a potentially lethal phenomenon that must require strong regulatory mechanisms in order to avoid uncontrolled cell death. Figure 4 represents some of the events that are involved in the response of macrophages to ATP_o and the proposed mechanism that may be important to regulate the permeabilization phenomenon. According to our results, the fast-activating depolarizing current ($P2X_7$ channel), the delayed Ca^{2+}-dependent K^+ hyperpolar-

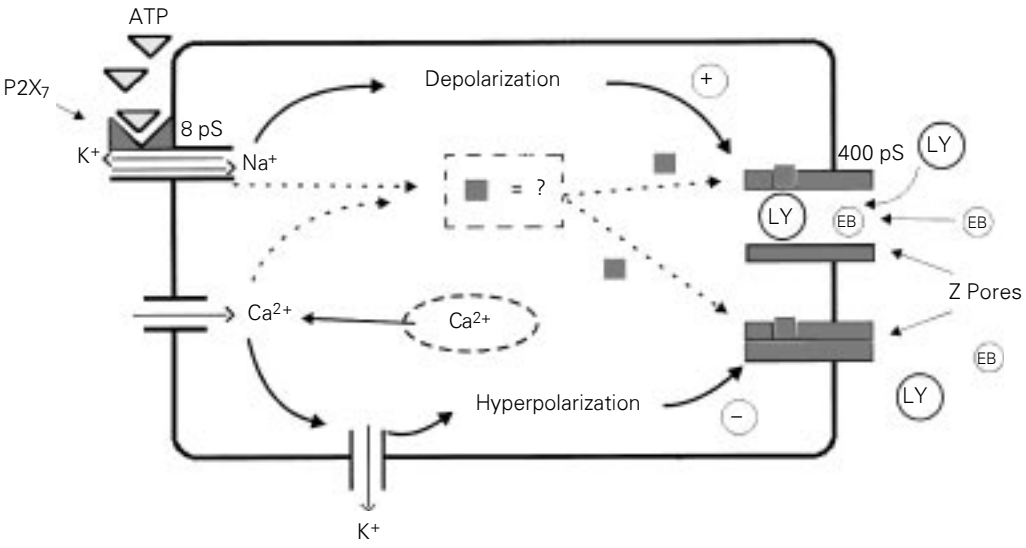

Figure 4 - Regulation of the P2Z-associated ATP_o-induced permeabilization: a hypothesis. In this simplified model, P2Z-associated phenomena are represented. For more details see the last section of the text. EB = Ethidium bromide; LY = Lucifer yellow.

izing current and the permeabilization phenomenon (Z pores) are probably distinct phenomena. The opening of the voltage-dependent Z pores involves the mobilization of as yet unidentified second messengers and the depolarization induced by the ligand-gated cation channel $P2X_7$. In opposition to this mechanism, the simultaneous opening of Ca^{2+}-dependent K^+ channels will move the membrane potential towards more negative values, forcing Z pores to shut down. Intracellular Ca^{2+} will initially increase due to at least two mechanisms: release from intracellular stores and opening of Ca^{2+} channels. P2Z (shown as $P2X_7$ channel) and other P2 purinoceptors such as P2Y and P2U (not shown) may be important at this stage. The final outcome of this process will depend on the relative intensity of each event. A dominance of the Ca^{2+}-dependent K^+ channels will keep the cell in a hyperpolarized state and avoid membrane permeabilization. On the other hand, a dominance of $P2X_7$ currents will depolarize the cell and favor the opening of Z pores and membrane permeabilization.

The elucidation of the physiological role of extracellular ATP requires the study of the expression and activity of all receptors and channels involved in P2-dependent phenomena. The use of electrophysiological and other biophysical tools will continue to provide new clues for the understanding of the possible immunoregulatory role of these purinoceptors.

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