P2Z purinoceptor-associated pores induced by extracellular ATP in macrophages and J 774 cells

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Coutinho-Silva, Robon, and Pedro Muanis Persechini. P2Z purinoceptor-associated pores induced by extracellular ATP in macrophages and J 774 cells. Am. J. Physiol. 273 (Cell Physiol. 42): C1793–C1800, 1997.—Millimolar concentrations of extracellular ATP (ATP), can induce the permeabilization of plasma membranes of macrophages and other bone marrow-derived cells to low-molecular-weight solutes, a phenomenon that is the hallmark of P2Z purinoceptors. However, patch-clamp and whole cell electrophysiological experiments have so far failed to demonstrate the existence of any ATP,–induced P2Z-associated pores underlying this permeabilization phenomenon. Here, we describe ATP,–induced pores of 409 ± 33 pS recorded using cell-attached patch-clamp experiments performed in macrophages and J 774 cells. These pores 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(hydroxymethyl)aminomethane, N-methyl-D-glucamine, and glutamate; their opening is favored at temperatures higher than 30°C; they are blocked by oxidized ATP and Mg; and they can be triggered by 3′-O-(4-benzoylbenzoyl)-ATP but not by UTP or ADP. We conclude that the pores described in this report are associated with the P2Z permeabilization phenomenon.

IT HAS BEEN WELL ESTABLISHED that extracellular ATP (ATP) may trigger intracellular signaling pathways, open ion channels, and induce different physiological responses depending on cell type and P2 purinoceptors expressed [reviewed by Dubyak and El-Moatassim (12)]. Five types of receptors, termed P2Y, P2U, P2X, P2T, and P2Z, have been identified based on pharmacological and functional studies (7, 15). Recently, based mainly on the analysis of protein sequences and signal transduction mechanisms, a new nomenclature scheme has been proposed for these receptors (5, 15, 36). According to this view, there are two major families of P2 purinoceptors, one with the properties of intrinsic ion channels, termed P2X, and the other coupled to G proteins, termed P2Y.

The term P2Z continues to be used to name receptors associated with the opening by ATP of a nonselective, poorly characterized ion pore (11, 15). Its presence has been described in many tissues and systems, including bone marrow-derived cells such as macrophages, mast cells, thymocytes, some lymphocytes, the phagocytic cells of the thymic reticulum (PT-R cells), and Langerhans cells (9, 11, 27, 38, 39). P2Z receptors have been frequently detected indirectly by the permeabilization of the cell membrane to fluorescent dyes such as Lucifer yellow and ethidium bromide that happens a few minutes after ATP, addition (34, 39). Macrophages and mast cells are permeable to solutes of up to 900 Da, whereas some lymphocytes, thymocytes, and hematopoietic stem cells are permeable to solutes of up to 400 Da (12, 15, 27).

The physiological function of P2Z purinoceptors in the immune system is still an open question (11, 12, 29). In macrophages, it has been associated with interleukin-1 maturation and release (21), formation of multinucleated giant cells (14), and elimination of macrophages infected by intracellular parasites (22). However, due to the strong permeabilization phenomenon and the induction of apoptosis in some cell types such as thymocytes and macrophages, a role in cell death has been proposed (11).

Patch-clamp studies have generated valuable information regarding the interaction of ATP with P2Z purinoceptors. However, experiments performed in mast cells (38) and macrophages (1, 6, 18) have so far failed to detect single-channel currents that could be associated with an ATP,–induced pore. In macrophages and PT-R cells, two currents can be promptly induced by ATP, a depolarizing current that is selective for small monovalent cations and a Ca,–dependent K, current (1, 9, 17). We have recently shown that this depolarizing current can be ascribed to a 5–to 8-pS channel that is too small to explain the permeabilization phenomenon (10). Moreover, the proposal of involvement of hemi-gap junction channels formed by connexin-43 (3) could not be confirmed (2).

To further investigate the nature of the P2Z-associated permeabilization phenomenon, we performed experiments using the cell-attached configuration of the patch-clamp technique in macrophages under conditions known to induce permeabilization. These experimental conditions would avoid modifications of the intracellular environment and increase our chances of obtaining direct electrophysiological recordings of a putative P2Z pore. Here, we describe for the first time single-channel currents of large nonselective channels opened by ATP, in mouse peritoneal macrophages and J 774 cells. The conductance, selectivity, and pharmacological characteristics of these pores are consistent with the expected properties of a P2Z-associated pore.

MATERIALS AND METHODS

Cells. Thioglycollate-elicited macrophages were obtained from the intraperitoneal cavity of Swiss-Webster mice. Cells were transferred to RPMI 1640 medium containing 5% heat-inactivated fetal calf serum, 2 g/l sodium bicarbonate, 100 U/ml penicillin, and 100 µg/ml streptomycin and were plated in 35-mm petri dishes. All surgical manipulations were performed under aseptic conditions.
were performed under ether anesthesia. After 1 h of incubation at 37°C in a 5% CO2 humidified atmosphere, nonadherent cells were removed, and the adherent cells were kept in the same conditions for 4 h to 15 days until use. Unless otherwise specified, we used macrophages in our experiments. In some experiments, the mouse macrophage J774-M cells were used. The cells were grown in 25-cm2 tissue culture flasks kept at the same conditions as above and were plated in 35-mm petri dishes for 2 to 4 days before use. Reagents: ATP, UTP, ADP, 3'-O-(4-benzoylbenzoyl)-ATP (BzATP), oxidized ATP, ethylene glycol bis(β-aminoethyl ether)-N,N',N' -tetraacetic acid (EGTA), tri(hydroxy methyl)aminoethane (Tris), N-methyl-d-glucamine (NMDG), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and sodium glutamate were purchased from Sigma Chemical (St. Louis, MO). The pore-forming protein of cytotoxic lymphocytes (perforin) was purified from the CTLL-R8 cell line by column coupled to fast-performance liquid chromatography (Pharmacia Biotech, Uppsala, Sweden) as previously described (20, 30). Perforin activity (5 hemolytic U/µl) was quantified in a Ca2+-dependent hemolytic assay as described previously (20).

Electrophysiological measurements. Ionic currents were studied in cell-attached and, in some cases, whole cell configuration, using an EPC-7 amplifier (List Electronic, Darmstadt, Germany) according to standard patch-clamping techniques (16). The petri dishes containing the cell were filled with 10 ml of solution and were placed in the heated stage of a microscope. Gigaohm seals were formed after offset potential compensation, using heat-polished micropipettes of 5–10 MΩ. ATP, UTP, and ADP (100 mM) were applied to 10 ml of extracellular solution by manually dropping 50–100 µl of solution into the plate dish. BzATP (100 µM, 14 mM) was applied to 7 ml of extracellular solution. All drugs were diluted in normal extracellular solution and were stored in the dark at –20°C until use. Temperature was continuously monitored with a digital thermometer placed in the extracellular solution and, unless otherwise specified, was maintained in the range of 30–37°C.

Current and voltage were simultaneously recorded on a paper chart recorder (Mark VII WR 3310; Graphtec, Yokohama, Japan) and in a VCR tape, and digitalization was performed by a Neurocorder (model DR-390; Neuro Data Instruments) for off-line analysis. Conductances of unitary events and then measuring the amplitude. Curves 1–6 are representative records of each type of experiment described in the text. I-V plots in Figs. 1–3 were taken from single channels of the same patches. Mean values and the number of experiments (n) are described in the text and in Table 1. Current signals were filtered at 3 kHz during acquisition and at 300 Hz during the play back of the recordings. To correct for junction potentials, the ground electrode was placed in a chamber containing the same solution as the patch pipette and was connected to the extracellular solution by an agar bridge. The diffusion potentials at the tip of the patch pipette were then measured for each intrapipette solution at the beginning of each experiment, as described by Neher (26). All voltages shown in Figs. 1–6 are relative to the holding potentials (Vh) inside the patch pipette, without any corrections, whereas the numeric values of the reversal potentials (Vrev) shown in the text and in Table 1 are corrected for the pipette junction potential. Average data are given as means ± SD.

RESULTS

Single-channel currents induced by ATPp in cell-attached patches. Because previous patch-clamp experiments have failed to detect P2Z-associated pores (1, 18, 24, 25), we decided to investigate the occurrence of any ATPp-triggered ion channel in cell-attached patches at 30–37°C using normal extracellular solution both in the patch pipette and in the extracellular bath. These conditions avoided any disturbance to the intracellular medium and assured that most macrophages would become permeable to lucifer yellow upon ATP addition. Moreover, because the transduction signals involved in the permeabilization phenomenon may require second messengers (4), ATP was added to the extracellular solution only after gigaseal formation. As shown in Fig. 1A, addition of normal extracellular solution in the proximity of the cell did not induce any ion channel activity on the patch, whereas addition of ATP induced the opening of several ion channels. At pipette Vh ranging from –20 to –60 mV, larger steps of current were frequently observed. To evaluate channel conductances and Vrev, the resting transmembrane potential of the cell was measured in independent current-clamp whole cell experiments performed at least 30 s after addition of ATPp. Using normal intracellular solution in the pipette, we obtained a value of –1 ± 2 mV (n = 6), consistent with the already described depolarization induced by ATPp (1, 6, 18).

A preliminary evaluation of our data showed that the observed channels belonged to at least two categories: those displaying Vrev compatible with K+ channels and those with Vrev close to 0 mV and conductances larger than 300 pS. We have therefore decided to focus our attention on the latter. To decrease the presence of unrelated channels, we have always waited 3–5 min after obtaining the cell-attached seal before applying ATP. Patches displaying spontaneous channel activity were discarded. In some experiments, we also added 2.5 mM Ba2+ to both intrapipette and extracellular solutions to block K+ channels. However, the use of ion channel inhibitors was avoided because we were looking for a pore of unknown properties and we did not.
Similar values were obtained for J774 cells (409 ± 6 pS, 2 ± 3 mV, n = 3). Addition of 2.5 mM BaCl2 to both intrapipette and extracellular solutions did not significantly change these values (468 ± 52 pS, −0.6 ± 0.6 mV, n = 3).

We have observed channel activity similar to that described in Fig. 1 in 87% of cell-attached patches (n = 80). The first signs of channel activity were detected 10–180 s after ATPo application. Frequently, two or three channels stayed open at the same time. Opening times were widely scattered, varying from 60 ms to 30 s, and were dependent on membrane potential, as shown later (e.g., see Fig. 5).

The fact that ATP permeabilizes the whole macrophage membrane, inducing osmotic stress and disruption of the intracellular homeostasis, raises the question of whether the pores we have described are triggered by specific P2Z-associated signaling cascades or represent a nonspecific effect that could be activated even by other nonnucleotide permeabilizing agents. To address this question, we added perforin to macrophages during cell-attached experiments performed under the same conditions used for ATP. This pore-forming protein is derived from cytotoxic lymphocytes and can open large pores ranging from 400 pS to nS in the membranes of many cell types (31). Pore insertion takes <1 min; therefore, the time course of permeabilization by perforin is in the same order of magnitude as by ATP. Addition of 250 hemolytic units of perforin to cellassociated macrophages (n = 6) did not induce the opening of any large-conductance channels in the macrophage patches (data not shown). However, a pattern of cell blebbing, typical of perforin pore formation, could easily be observed in the neighboring cells within 1–2 min, as previously described (31). Pore formation by perforin was further confirmed in whole cell recordings in which a series of step-like conductance increases typical of perforin pore formation was observed a few seconds after perforin addition (data not shown). Moreover, in another series of experiments, perforin was shown to induce permeabilization of macrophages to

want to disturb any of the cell characteristics at this stage of the investigation.

Figure 1, B and C, illustrates the opening of large channels observed at different Vm in a macrophage and in a J 774 cell, respectively. I-V plots of these channels are shown in Fig. 1D. The mean conductance and Vrev values measured in seven different macrophages were 409 ± 33 pS and 2 ± 2 mV, respectively (Table 1). Similar values were obtained for J 774 cells (409 ± 64

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Fig. 1. Single-channel activity induced by extracellular ATP (ATPo) in cell-attached patches of macrophages and J 774 cells. Experiments were performed at 30–37°C, using normal extracellular solution inside the pipette. A: typical recording from a macrophage indicating that addition of normal extracellular solution (first two vertical arrows) did not cause any effect, whereas addition of ATP (third vertical arrow) caused an inward current and unitary channel activity followed by the opening of large current steps. Holding pipette potential (Vh) was −30 mV. Large-conductance channels are recorded at different Vm values in a macrophage (B) and in a J 774 cell (C). Correspondent current-voltage (I-V) plots are shown in D. Conductance and reversal potentials (Vrev), calculated by linear regression, were 402 pS and 0.2 mV for the macrophage and 422 pS and −0.7 mV for the J 774 cell. Final concentration of ATPo was 500 μM in all experiments. Horizontal arrows on left indicate I = 0 pA, and values of potential on right of each record refer to Vh, without correction of junction potentials.

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Table 1. Reversal potential and conductance of ATPo-induced pore

<table>
<thead>
<tr>
<th>Pipette Solution</th>
<th>Cell</th>
<th>Agonist</th>
<th>Vrev, mV</th>
<th>Conductance, pS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal extracellular</td>
<td>Macrophage</td>
<td>ATP</td>
<td>2 ± 2 (7)</td>
<td>409 ± 33 (7)</td>
</tr>
<tr>
<td>J 774 cells</td>
<td></td>
<td></td>
<td>2 ± 3 (3)</td>
<td>409 ± 64 (3)</td>
</tr>
<tr>
<td>Macrophage</td>
<td>BzATP</td>
<td></td>
<td>−0.8 ± 2 (4)</td>
<td>425 ± 22 (4)</td>
</tr>
<tr>
<td>NMDG</td>
<td>Macrophage</td>
<td>ATP</td>
<td>−2.3 ± 2.5 (8)</td>
<td>350 ± 30 (8)</td>
</tr>
<tr>
<td>Tris</td>
<td>Macrophage</td>
<td>ATP</td>
<td>−1 ± 4 (3)</td>
<td>340 ± 20 (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>−1 ± 5 (6)</td>
<td>221 ± 31 (6)*</td>
</tr>
<tr>
<td>Glutamate</td>
<td>Macrophage</td>
<td>ATP</td>
<td>1.3 ± 1.2 (3)</td>
<td>358 ± 78 (3)</td>
</tr>
<tr>
<td>Low NaCl</td>
<td>Macrophage</td>
<td>ATP</td>
<td>2 ± 2 (3)</td>
<td>345 ± 31 (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5 ± 2 (4)</td>
<td>279 ± 8 (4)*</td>
</tr>
</tbody>
</table>

Values are means ± SD; no. of experiments in parentheses. NMDG, N-methyl-D-glucamine; BzATP, 3’-O-(4-benzoylbenzoyl)-ATP. Reversal potentials (Vrev) were obtained from current-voltage curves and were corrected for pipette junction potentials as described in Materials and Methods. Values refer to conventional transmembrane potential, assuming that the cells have reached zero transmembrane potential after extracellular ATP (ATPo) application. * Larger and smaller conductances were compared in each extracellular solution; P < 0.01 according to Student's t-test.
ethidium bromide and lucifer yellow (data not shown). These results demonstrate that perforin induces pore formation only in the portion of membrane situated outside the cell-attached patch. Our data are consistent with the interpretation that the ATP-induced pores are triggered by an ATP-specific mechanism and not by other mechanisms induced by unrelated permeabilizing agents.

Selectivity of ATP$_o$-induced channels. The size and the $V_{\text{Rev}}$ of the channels described in Fig. 1 suggested that they could be nonselective, allowing the passage of both cations and anions of different sizes, as it would be expected for a pore involved in the P2Z-associated permeabilization phenomenon. To investigate this question, we have performed cell-attached ion substitution experiments using four intrapipette solutions containing different concentrations of cations and anions. Junction potentials were taken into account as described in MATERIALS AND METHODS. In all solutions, we observed large channels displaying $V_{\text{Rev}}$ close to 0 mV, but, in some conditions, the channels had at least two different sizes (Figs. 2 and 3 and Table 1). When NMDG [relative molecular weight (M$_r$) of 195] substituted for Na$^+$ (Fig. 2A), a channel of $350 \pm 30$ pS ($n = 8$) and $V_{\text{Rev}}$ of $-2.3 \pm 2.5$ mV were observed. When Tris (M$_r$ 121) substituted for Na$^+$ (Fig. 2, B, C, and E), channels with two different conductances ($340 \pm 20$ pS, $n = 3$ and $221 \pm 31$ pS, $n = 6$) were observed. Both had $V_{\text{Rev}}$ close to 0 mV and could be clearly distinguished in I-V plots (Fig. 2E and Table 1).

When glutamate (M$_r$ 146) substituted for Cl$^-$ (Fig. 2D), the recordings were frequently unstable, but all cells tested ($n = 12$) responded to ATP$_o$. We could plot I-V curves for the channel activity of three of these cells, obtaining the values of $358 \pm 78$ pS and $1.3 \pm 1.2$ mV for the conductance and the $V_{\text{Rev}}$, respectively.

The data described so far are consistent with channels that are nonselective for both cations and anions. However, channels selective for either cations or anions could lead to similar results. Therefore, we have decreased NaCl concentration to 34 mM in the intrapipette solution, using mannitol to maintain osmolarity, a condition that makes cation and anion channels have $V_{\text{Rev}}$ values of opposite signs (33). As shown in Fig. 3 and in Table 1, activities of two channels with conduc-
stances of 345 ± 31 (n = 3) and 279 ± 8 (n = 4) were found. $V_{\text{Rev}}$ values were 2 ± 7 mV (n = 3) and 5 ± 2 (n = 4) mV, respectively, leading us to the conclusion that both channels are cation and anion nonselective pores triggered by ATP$_o$.

The finding that the above-described pores are permeable to large cations and anions such as Tris, NMDG, and glutamate suggests that they could be involved with the phenomenon of P2Z-associated permeabilization, with a size exclusion limit of at least 195 Da. Therefore, we named the larger and smaller pores $Z-1$ and $Z-2$, respectively.

Other properties consistent with a P2Z-associated pore. To further characterize the above-described pore(s) as involved in the P2Z-associated permeabilization, we investigated other known properties of this phenomenon (7, 11, 12, 23, 34): temperature dependence, triggering by BzATP, lack of effect of ADP and UTP, and blockade by Mg$^{2+}$ and oxidized ATP (Fig. 4). In this series of experiments, we kept the intrapietette $V_H$ in the range from −20 to −30 mV since, under this condition at 37°C, ATP$_o$ induces pore opening in <30 s, as shown in Fig. 1.

At 18–22°C, the cell-attached patches remained silent for >10 min after ATP$_o$ addition (Fig. 4A, top trace). However, upon gradual heating, $Z-1$ type channels could be easily observed in all cells studied (n = 5) when the temperature reached 30–37°C (Fig. 4A, bottom trace).

BzATP, a P2Z agonist that is more potent than ATP in inducing permeabilization and Ca$^{2+}$ mobilization (13), was also able to open large-conductance channels with I-V curves similar to the $Z-1$ pores triggered by ATP$_o$ (Fig. 4B). The mean values of the conductance and $V_{\text{Rev}}$ were 425 ± 22 pS (n = 4) and −0.8 ± 2 mV (n = 4), respectively. In addition, neither ADP (n = 6) nor UTP (n = 6) induced pore activity in the macrophage patches (Fig. 4, C and D, respectively).

Another characteristic of P2Z-associated permeabilization, the requirement of ATP$^{4-}$, was investigated by the addition of Mg$^{2+}$ to the extracellular solution. No pores were detected when ATP was added to cells kept in normal extracellular solution containing 10 mM Mg$^{2+}$ (Fig. 4E, n = 5). Under this experimental condition, the permeabilization of macrophages to ethidium bromide was also blocked (data not shown). Furthermore, addition of MgCl$_2$ (30–100 µL of a 1 M solution) during cell-attached recordings induced the closing of the pores previously opened by ATP within the following 40 s (Fig. 4F, n = 6).

Pore formation was also inhibited by preincubation of macrophages with 300 µM oxidized ATP for 2 h at 37°C (Fig. 4G, n = 6). In parallel experiments, we have confirmed that, under this same condition, the ATP-induced permeabilization to ethidium bromide was also inhibited (data not shown). In two of the experiments with oxidized ATP (e.g., Fig. 4G), we could detect a transient current just after ATP addition. However, this current did not correspond to the opening of large pores as the ones observed after the addition of ATP$_o$ in the absence of oxidized ATP in a similar experiment (Fig. 4H).

Regulation by voltage. The activity of $Z-1$ and $Z-2$ channels was modulated by voltage. Opening times of several seconds could be measured at negative $V_H$ values, whereas, at positive values, both pores closed. This is evident in Fig. 5 in which BzATP was applied to a macrophage. A membrane patch that would look otherwise like a leaky seal at $V_H$ = −9 mV displayed successive step-like current decreases, returning to 0 pA at +30 mV (Fig. 5B). Then, the patch remained silent for 3 min until $V_H$ was shifted back to negative values, when a $Z-1$ pore opened again (Fig. 5D). Intra-
smaller than 500 µM. However, when the final ATP concentration was obtained by adding concentrated ATP to the extracellular solution, activity, like the ones described in Figs. 1–5, was observed in these cells, but no single channels of pores were recorded from P2X7-transfected cells (37).

In the search of possible reasons for the lack of electrophysiological data corresponding to an ATP-induced pore, we performed cell-attached patch-clamp experiments in macrophages submitted to conditions known to induce permeabilization. Here, we describe the presence of large nonselective channels in macrophages and J774 cells. The finding that these channels are permeable to large cations and anions such as Tris, NMDG, and glutamate (M, 121–195 Da) suggests that they are involved with the phenomenon of P2Z-associated permeabilization. Therefore, we named them Z-1 and Z-2 pores, for the larger and the smaller pores, respectively. In accordance with this conclusion is the fact that these ATP-induced pores display several other properties of the P2Z-associated permeabilization (7, 11, 12, 34; see Fig. 4); they are temperature dependent and are not triggered by ATP in the presence of high extracellular Mg2+ concentration, indicating the requirement for ATPγS. Moreover, BzATP, but neither ADP nor UTP, can substitute for ATP in the induction of pore activity. In addition, oxidized ATP, an

DISCUSSION

The P2Z purinoceptor-associated permeabilization phenomenon was described in macrophages and mast cells more than 10 years ago (8, 35, 38). However, all whole cell and single-channel currents described so far in these cells do not have the characteristics expected for a cation and anion nonselective pore. A protein called P2X7 has been recently cloned and displayed the pharmacological and functional characteristics of P2Z receptors when expressed in several cell lines (37). ATP-induced membrane permeabilization and a cation current similar to the ones present in macrophages were observed in these cells, but no single channels of pores were recorded from P2X7-transfected cells (37).

Cascade pattern of channel opening. Unitary channel activity, like the ones described in Figs. 1–5, was obtained by adding concentrated ATP to the extracellular bath in doses designed to reach a final concentration smaller than 500 µM. However, when the final ATP concentration was 1 mM or higher, it was frequent to observe rapid and successive increases in the current, eventually leading to saturation of the amplifier, a condition usually associated with seal disruption. However, in eight patches, most of which had previously displayed unitary Z-1 pore activity, we could distinguish four to seven independent steps before saturating the amplifier (Fig. 6). The whole process lasts < 1 s. Due to the time course and the uncontrolled nature of these events, an accurate measurement of the Vrev of these steps was not possible to obtain. However, examining the events of different cells, it is reasonable to assume that the Vrev would be close to 0 mV as in the case of the Z-1 unitary channel. Under this assumption, the current steps observed using normal extracellular solution would correspond to conductances ranging from 461 to 772 pS (589 ± 128 pS, n = 8).

![Fig. 5. Regulation by voltage. A: macrophage patch displayed large-conductance single channels after addition of 200 µM BzATP (final concentration). B: same patch continued to increase conductance and was kept at Vh = −9 mV for a few seconds until a ramp of potential was applied, shifting Vh to +30 mV (vertical arrows). Soon after Vh reached +30 mV, conductance returned to 0 in 5 successive steps. C: no channel activity was recorded in the same patch, whereas Vh remained at +30 mV. D: large-conductance, Z-1 channel opened again when Vh was shifted to −28 mV. Horizontal arrows on left indicate I = 0 pA, and values of potential on right of each record refer to Vh, without correction of junction potentials.](image)

![Fig. 6. Cascade of step-like conductance increase. Macrophage patch that had previously displayed large-conductance (Z-1) single-channel activity after addition of ATPγS suddenly increased conductance in 7 successive steps until saturation of the recording system. Event happened 6.5 min after addition of ATPγS (1 mM final concentration). Horizontal arrow on left indicates I = 0 pA, and value of potential on right refers to Vh, without correction of junction potentials.](image)
inhibitor of the permeabilization phenomenon, also inhibits pore activity. Taken together, our results lead to the conclusion that Z pores are involved in the P2Z-associated permeabilization phenomenon. However, more experiments are needed to establish the size limit of the molecules that can diffuse through these pores and compare it with the know values of the permeabilization phenomenon.

In several membrane patches, other channels could be observed under conditions in which Z pores were not opened (e.g., Fig. 4G). This observation could be explained by the activation of ATP-induced channels via other P2 receptors present in the macrophage membrane. Two possibilities are the activation of Ca\(^{2+}\)-dependent K\(^+\) channels (1, 17) and the ATP-activated Ca\(^{2+}\)-permeable channel described by Naumov et al. (24, 25). In this regard, it should be noticed that oxidized ATP does not inhibit the ATP-induced increase of the intracellular Ca\(^{2+}\) concentration (23).

Only Z-1 pores were clearly present when normal extracellular solution was used in the patch pipette (Fig. 1 and Table 1). Its conductance (409 pS) is in accordance with the expected value of a pore permeable to lucifer yellow (\(M_r\) of 457 and Stokes radius of 7.8 Å; see Refs. 32, 38). Z-2 pores were more evident in solutions with low Na\(^+\) and/or low Cl\(^-\) concentration. However, although data were not always enough to plot I-V curves, single-channel activity or steps of current compatible with Z-2 pores were also observed under all conditions studied here (data not shown). It is not clear at the moment whether Z-2 represents an independent channel or a subconductance state of the Z-1 pore. However, the existence of two P2Z-associated pores or opening states is consistent with data showing that, although macrophages are permeable to molecules of up to 900, some lymphocytes seem to have a molecular weight cut-off of ~400.

The observation that the Z pores opened in membrane patches isolated from the ATP-containing extracellular medium by the gigaohm seals indicates that these pores are coupled to purinoceptors by a pathway involving second messengers. This conclusion is consistent with previous experiments that failed to obtain large conductance steps in whole cell experiments in which the intracellular milieu was not preserved (1, 18). We have not elucidated the nature of the second messengers involved in the opening of Z pores. However, it is interesting to note that recent evidence suggests the involvement of calmodulin and phospholipase D in the permeabilization phenomenon (4, 19, 28).

On the other hand, it has been demonstrated that the P2Z/P2X\(_7\) receptor is a ligand-gated channel associated with a cation current that displays a single channel conductance of 5–8 pS, not directly involved in the transport of low-molecular-weight solutes (1, 10, 12, 37). These results can be conciliated with the second-messenger hypothesis by proposing that, although Z pores are activated by the P2Z/P2X\(_7\) receptor, they are distinct channel proteins. Alternatively, Z pores could be a new (second messenger-dependent) activation state of the P2Z/P2X\(_7\) receptor itself.

There is indeed some evidence in the literature indicating that permeabilization can be separated from other P2Z-associated phenomenon: differential activation of the cation current and the nonselective pores can be achieved in Xenopus oocytes expressing macrophage mRNA (28); calmodulin antagonists are able to prevent the lytic effects of ATP\(_o\) without affecting calcium influx and membrane depolarization (4); and, in P2X\(_7\)-transfected cells, permeabilization, but not the cation current, is dependent on the cytoplasmatic tail of the P2X\(_7\) protein (37). The full elucidation of this problem will require the identification of the intracellular pathways triggered by P2Z receptors and the cloning of the permeabilization pore(s).

One interesting property of Z-1 and Z-2 pores is that they tend to be closed at negative transmembrane potential (positive \(V_{in}\)). This finding suggests that, in macrophages, they can be regulated by the balance of two opposing mechanisms also induced by ATP\(_o\): the fast depolarization caused by the small cation channels recently described by us (10) and the delayed and transitory Ca\(^{2+}\)-dependent K\(^+\) current that follows the first one (1, 17, 29). These same mechanisms would also regulate the P2Z-associated permeabilization phenomenon. In this regard, it is interesting to notice that high extracellular K\(^+\) concentration, a condition that depolarizes the cells, enhances permeabilization in peripheral blood lymphocytes (Ref. 39 and our unpublished observations).

The cascade of steps shown in Fig. 6 is possibly associated with Z-1 pores and permeabilization. However, more data are needed to clearly establish this connection. The explosive nature of the events suggests a cooperative phenomenon in which the opening of a first pore facilitates the opening of the next ones. This pattern of conductance increase has already been described for the insertion of pores of perforin in cell membranes (31).

Our results suggest that the Z pores described in this report are triggered by P2Z purinoceptors and are involved in the permeabilization of the macrophage plasma membrane to low-molecular-weight solutes. The study of these pores may contribute to the understanding of the mechanism and functional role of plasma membrane permeabilization induced by ATP\(_o\) in macrophages and other cells.

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