Pharmacological properties of a pore induced by raising intracellular Ca²⁺

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Faria RX, Reis RA, Casabulho CM, Alberto AV, de Farias FP, Henriques-Pons A, Alves LA. Pharmacological properties of a pore induced by raising intracellular Ca²⁺. Am J Physiol Cell Physiol 297: C28–C42, 2009. First published March 25, 2009; doi:10.1152/ajpcell.00476.2008.—Recent studies on the P2X7 receptor in 2BH4 cells and peritoneal macrophages have demonstrated that the raise in intracellular Ca²⁺ concentration induces a pore opening similar to P2X7 receptor pore. Herein, we have investigated whether the pore activated by the elevation of intracellular Ca²⁺ concentration is associated to P2X7 receptor. Using patch clamp in cell attached, whole cell configuration, and dye uptake, we measured the pore opening in cell types that express the P2X7 receptor (2BH4 cells and peritoneal macrophages) and in cells that do not express this receptor (HEK-293 and IT45-R1 cells). In 2BH4 cells, the stimulation with ionomycin (5–10 μM) increased intracellular free Ca²⁺ concentration and induced pore formation with conductance of 421 ± 14 pS, half-time (t½) for ethidium bromide uptake of 118 ± 17 s, and t½ for Lucifer yellow of 122 ± 11 s. P2X7 receptor antagonists did not block these effects. Stimulation of HEK-293 and IT45-R1 cells resulted in pore formation with properties similar to those found for 2BH4 cells. Connexin hemichannel inhibitors (carbenoxolone and heptanol) also did not inhibit the pore-induced effect following the increase in intracellular Ca²⁺ concentration. However, 5-(N,N-hexamethylene)amiloride, a P2X7 receptor pore blocker, inhibited the induced pore. Moreover, intracellular signaling modulators, such as calmodulin, phospholipase C, mitogen-activated protein kinase, and cytoskeleton components were important for the pore formation. Additionally, we confirmed the results obtained for electrophysiology by using the flow cytometry, and we discarded the possibility of cellular death induced by raising intracellular Ca²⁺ at the doses used by using lactate dehydrogenase release assay. In conclusion, increased concentration in intracellular Ca²⁺ induces a novel membrane pore pharmacologically different from the P2X7 associated pore and hemigap-junction pore.

P2X7 receptor; pore formation; second messenger

P2X receptors are members of a family of P2 receptors with seven subtypes (P2X1-P2X7) that induce an increase in intracellular Ca²⁺ concentration when activated. Among the P2X receptors, P2X7 subtype is the most divergent component of this family in terms of pharmacological properties, molecular structure, and function (54). This receptor forms a nonselective cation channel upon low ATP concentration, whereas at high ATP concentration, this receptor induces a pore formation that allows the flux of molecules of up to 900 Da and may lead to cell death. Although P2X7 receptor is associated with expression of both functional channels and large pores, the structure of the large pore-forming subunits and whether the channel and the large pore are the same structure are still not known.

From a physiological point of view, P2X7 is mainly expressed in the immune system found in all cells of this system studied so far. The activation of P2X7 receptor has several effects in the immune system such as release of mature interleukin-β and other pro-inflammatory cytokines (interleukin-18, tumoral necrosis factor) by macrophages and dendritic cells (30, 39, 53); degranulation of mast cell (38); killing of Mycobacterium tuberculosis inside human and mouse macrophages; and apoptosis, depending on the concentration of ATP applied (33, 71).

Although the P2X7 itself is considered as a nonselective large pore, some groups have had difficulty in detecting it in cells expressing the heterologous P2X7 receptor, suggesting that the pore-forming molecule might be a distinct entity (11, 14, 77). Similarly, some cell types present a native P2 receptor that shares most of the P2X7 receptor pharmacological features but fail to permeabilize (43, 62). In keeping with this idea, Coutinho-Silva and Persechini (16) described a pore with a unitary conductance of 409 pS and a pharmacological profile similar to P2X7 pore (16). In that study, pore formation was detected only in cell-attached configuration, which maintains the intracellular milieu. Thus the authors suggested that this pore might not be associated directly to P2X7/P2X7 but activated by a second messenger.

The mechanisms underlying the P2X7 pore formation are unknown, but at least two hypotheses have been postulated. In one view, the channel formed by P2X7 receptor is gradually open upon activation, increasing its permeability from small molecules to large ones (up to 900 Da) (80, 81). In the other, P2X7 channel is formed upon activation, inducing the production or release of a second messenger that may autoactivate P2X7 receptor or an independent pore-forming membrane protein (16, 61). Recently, we have demonstrated that the action of second messengers is necessary to form the pore (28). Moreover, our data indicated that there was actually a pore associated to P2X7, i.e., the P2X7 channel is different from the low-selective pore. In keeping with our data, Jiang and co-workers (41) have elegantly reinforced that P2X7 channel is different from the low-selective pore associated with P2X7 activity. In addition, it was recently suggested that while P2X7 receptor is the protein responsible for the small conductance channel activity (8 pS), pannexin 1 is associated to dye uptake induced by P2X7 receptor (pore formation) (59). In another article (48), pannexin 1 was demonstrated as the molecular substrate for the pore formation (or cell death) in P2X7 receptor signaling complex.

Several groups have also found membrane pores with characteristics similar to P2X7-associated pore. Previously, Schilling and colleagues (66) showed that maitotoxin activates a...
cytolytic pore with characteristics similar to P2X7. Recently, several groups have shown that hemi-gap junctional channels have some molecular size cut-off properties similar to P2X7 pore (9, 15).

In this study, our group has found a large pore induced by elevation of intracellular Ca$^{2+}$ similar to P2X7 receptor pore. This prompted us to investigate the properties of this pore induced by raising intracellular Ca$^{2+}$. To address this issue, we have used patch-clamp technique in cell attached and whole cell configurations associated with dye uptake and flow cytometry.

MATERIALS AND METHODS

Cell Cultures

The thymic epithelial cell line (TEC) 2BH4, derived from C57BL/6 mouse thymus, was kindly provided by Dr. J. G. Amarantes-Mendes (University of São Paulo, Brazil). Human embryonic kidney (HEK-293) cells were obtained from Federal University of Rio de Janeiro Hospital, Cell Bank, Cryopraxis, Rio de Janeiro, Brazil. The Wistar rat-derived TEC line IT45-R1 was obtained from Dr. Tsunetoshi Itoh (Tohoku University, Sendai, Japan). These cells were cultured in RPMI 1640 medium containing 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) and plated on 35-mm culture dishes for 2–5 days.

Peritoneal macrophages were obtained from the intraperitoneal cavity of Swiss mice and prepared in our laboratory as previously described (28). Cells were incubated at 37°C in a 5% CO2 humidified atmosphere. Our protocols are in line with Ethical Principles in Animal Experimentation adopted by the Brazilian College of Animal Experimentation and were approved by Fiocruz Committee of Ethics in Research with the number (L-041/08).

Intracellular Ca$^{2+}$ Measurements

Cells were plated on glass coverslips (Biophysicsa Technologies, Sparks, MD) and loaded with 5 μM Fura-2-AM (Molecular Probes) for 1 h at room temperature in culture medium. The coverslips containing the cells were then washed and mounted in a three-compartment superfusion chamber. The central chamber containing the cells had a volume of 200 μl and was perfused with extracellular saline containing 1 mM CaCl$_2$ at a rate of 1 ml/min. The intracellular Ca$^{2+}$ concentration was monitored continuously at room temperature with a fluorescence photometer (Photon Technology, Princeton, NJ). Fura-2 was excited at 340 and 380 nm, and the emission at 510 nm with a fluorescence photometer (Photon Technology, Princeton, NJ). Dye uptake and ion currents were studied in the bath should completely depolarize the cell (solution B (in mM) consisted of 132 KCl, 5 NaCl, 1 CaCl$_2$, 1 MgCl$_2$, and 10 HEPES, pH 7.4; solution C (in mM) consisted of 150 LiCl, 5 KCl, 1 MgCl$_2$, 1 CaCl$_2$, and 10 HEPES, pH 7.4; solution D (in mM) consisted of 150 Tris, 5 KCl, 1 MgCl$_2$, 1 CaCl$_2$, and 10 HEPES, pH 7.4; solution E (in mM) consisted of 150 NMDG, 5 KCl, 1 MgCl$_2$, 1 CaCl$_2$, and 10 HEPES, pH 7.4; solution F (in mM) consisted of 150 KCl, 5 NaCl, 1 MgCl$_2$, 15 EGTA (14 mM EGTA-free bath solution), 1.43 CaCl$_2$ (4.8 mM Ca$^{2+}$-free pipette solution), and 10 HEPES, pH 7.4; solution H (in mM) consisted of 139 KCl, 5 NaCl, 1 MgCl$_2$, 8 EGTA (6 mM EGTA-free pipette solution), 1.93 CaCl$_2$ (14 mM Ca$^{2+}$-free pipette solution), and 10 HEPES, pH 7.4; solution I (in mM) consisted of 140 KCl, 5 NaCl, 1 MgCl$_2$, 3.4 EGTA (1 mg/mL EGTA-free pipette solution), 2.33 CaCl$_2$ (100 mM Ca$^{2+}$-free pipette solution), and 10 HEPES, pH 7.4. The solutions A, B, F, G, H, and I had osmolality checked with Vapor Pressuer Osmometer (model 5500, Welscor), and values ranged from 290 to 310 mosM.

Membrane Potential Control

Solution A was used inside the pipette and solution B in the bath to record single channel currents. The rationale for this procedure in the cell-attached configuration was twofold: 1) solution A inside the pipette warranted more physiological ion gradients across the patch so we could study more efficiently the ionomycin-activated phenomena; and 2) solution B in the bath should completely depolarize the cell membrane (~0 mV) so the real value of the voltage potential across the patch was the nominal holding potential applied. This circumvented the inconvenience of having to measure the cell potential to calculate the real holding potential.

Drug Application

Experiments were carried out under perfusion (RC-24 chamber, Warner Instrument, Hamdem, CT) to confirm data obtained through micropipette application. All drugs were dissolved in saline solution immediately before usage. Dye uptake and ion currents were studied by single application of agonists (from 5 to 30 s).

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Dye Uptake Assay

Cell permeabilization was visualized by the differential uptake of Lucifer yellow (457 Da, LY) and ethidium bromide (394 Da, EB) as previously described (73) or with rhodamine dextran (TRITC-Dextran; 3,000 Da). Dyes were dissolved in the bath or in the pipette solution (0.5 mg/ml LY or 0.25 μg/ml EB) according to a previously described protocol (73) and applied to the cell for 10 min at ~37°C. The culture dishes were then washed three times using normal saline (in mm: 150 KCl, 5 NaCl, 1 MgCl2, 0.1 EGTA, and 10 HEPES, pH 7.4) and observed under a fluorescence microscope (Nikon TE2000S, Nikon, Japan) equipped with rhodamine [excitation filter (EX): 540–580 nm and a barrier filter (BA): 600–660 nm] and fluorescein (EX: 450–490 nm BA: 520 nm) filters. To test the viability of the cells, trypan blue exclusion was performed during the seal and during the protocols. Trypan blue (0.4%) uptake was monitored (28) before the seal and at the end by using a light microscope. We used Kappa software (Kappa, Germany) for image analysis. One or two frames were acquired before agonist application (t = 0 s), and subsequent frames were obtained after cell stimulation in intervals of 30 s.

Some dye uptake experiments were done in whole cell patch-clamp configuration, and propidium iodide (PI) was added in the bath between 15 and 20 min after the establishment of the patch. Pipette solutions contained saline with distinct buffered Ca2+ concentrations (see saline solutions F, G, H, and I). The ionomycin pulse was applied 1–2 min after the PI addition. PI uptake was recorded until 5 min after the ionomycin stimulation.

Flow Cytometry-Based Analysis of Ca2+-Induced Pore Opening

Macrophages obtained from the intraperitoneal cavity of Swiss mice were prepared in our laboratory as previously described (28). Cells were incubated at 37°C in a 5% CO2 humidified atmosphere. Peritoneal macrophages were stimulated for 5, 15, 30, 45, or 60 min in the presence of pore formation inducers (ATP or ionomycin). In other experiments, cells were previously exposed to blockers and stimulated by ionomycin. Then 5 μg/ml PI was loaded on cells, and the samples were immediately analyzed by flow cytometry.

In the experiments with SYTO 13, cells were stimulated or not with agonists (ATP and ionomycin). Then PI (3 min) was loaded and SYTO 13 was added only at the moment of the measurement in the flow cytometer. SYTO 13 staining for flow cytometry was based on the procedure described by Schuurhuis et al. (67) and Sparrow and Tippett (72). Briefly, 1 × 10⁶ peritoneal macrophages in RPMI medium/10% FBS at 30–37°C were incubated with 4 nM SYTO 13 (final concentration) in a total volume of 0.2 ml for 1–5 min at 30–37°C. These assay conditions were determined to be optimal based on the results of extensive dose and time-course (dye loading) experiments.

Measurement of LDH Release

2BH4 or peritoneal macrophages monolayer cells (1 × 10⁶ cell/well) were incubated at 37°C for 1 h in the presence or absence of ionomycin. Cell lysis was quantified by measuring the release of LDH according to the manufacturer (Sigma). 2BH4 or peritoneal macrophages (5 × 10⁶) were incubated in 0.5 ml RPMI containing 1 mg/ml of bovine serum albumin at 37°C for 1 h. Cell supernatants were tested for LDH that reduces NAD+, which then converts a tetrazolium dye to a soluble, colored formazan derivative. The initial rate of absorbance was measured in a plate reader (Thermomax Microplate Reader, Molecular Devices, Menlo Park, CA) at a wavelength of 490 nm. Results represent the means ± SD of triplicate determinations.

Reagents

ATP, ionomycin, oxidized ATP (periodate-oxidized sodium salt), l-(N,N-bis[5-isoquinolinesulfonfonyl]-N-methyl-L-tyrosyl)-4-phenylpiperazine (KN-62), brilliant blue G (BBB), suramine, reactive blue-2 (RB-2), pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), 2',3'-O-(2',4',6'-trinitrophenyl)-ATP (TPN-ATP), citochalasin B, citochalasin D, colchicine, trifluoperazine, calmidazolium, 5-(N,N-hexamethylenyl)amiloride (HMA), acid 18α-glycercrhitinic, carbenoxolone, EB, forskolin, phorbol 12-myristate 13-acetate (PMA), LY, thapsigargin, A23187, rhodamine B isothiocyanate-dextran (RITC-Dextran), PD-98059, SB-20580, U-73122, U-73343, staurosporine, Ro-318220, calphostin C, YVAD, DEVD, LEHD, verapamil, and Cd2+ were purchased from Sigma Chemical (St. Louis, MO). Heptanol was purchased from Merck (Whitehouse Station, NJ). Trypan blue was obtained from Allied Chemical (Detroit, MI), and BAPTA-AM was obtained from Molecular Probes.
Data Analysis

Fluorescence data were normalized for the maximal value using Microsoft Excel, and results were plotted using GraphPad Prism version 3.0 (San Diego, CA).

Ca²⁺ concentration was calculated with Sliders v2.00 and WinMAXC version 2.10 software (Pacific Grove, CA). Data were expressed as means ± SD as indicated in the text. The statistical significance of differences between means was tested by one-way ANOVA followed by Tukey’s test. A two-tailed \( P < 0.05 \) was considered significant.

The open probability of the channel (\( P_o \)) in the presence or absence of ionomycin was calculated by dividing the mean open time by the sum of the mean open time and the mean shut times. Mean open and shut times were calculated by integration of the exponential components fitted to open- and shut-time distributions, respectively (83). Only recordings with single channel activity were considered to calculate \( P_o \). The single channel analysis was made in the Clampfit 9.0 software (Axon Instruments).

RESULTS

Rise of [Ca²⁺] in 2BH4 Cells Induced by Ionomycin and ATP

The effect of ionomycin on intracellular Ca²⁺ in the 2BH4 cells is shown in Fig. 1. Addition of 10 \( \mu \)M ionomycin induced Ca²⁺ changes with a biphasic response with a fast peak

Fig. 2. Single channel currents induced by ION in 2BH4 cells. A: single channel currents obtained on a cell attached patch from 2BH4 cells activated by 10 \( \mu \)M ION at different membrane potentials. B: mean current-voltage relationship for single channel currents. C: amplitude histogram of all single channel currents. Distribution was fitted by a sum of two Gaussians with means of 0.5 and \(-25\) pA. D: analysis of the channel amplitudes in relation to channel openings (\( P_o \) periods) recorded from a cell attached patch at \(-60\) mV in the 2BH4 cells. Values represent means ± SD of 10 independent experiments.
followed by a slower second phase that decreases with time (Fig. 1A). In the Fig. B, we applied 1 mM ATP on 2BH4 cells. After the treatment, as expected, there was a biphasic current with a fast peak followed by a slower second phase. As observed in Fig. 1, the amplitude of the Ca\(^{2+}\) peak induced by ionomycin was higher than the peak induced by ATP on 2BH4 cells. A similar pattern was obtained on peritoneal macrophages (Fig. 1, C and D).

**Ionomycin Induced Single Channel Large Conductance**

Figure 2 shows a representative recording trace from 2BH4 cells in the cell attached configuration exposed to 10 \(\mu\)M ionomycin. Figure 2A illustrates the qualitative effect of changing the membrane potential on the pore formation properties. Channel openings were, on average, 25. 3 ± 0.08 pA in amplitude at 60 mV. Around 36% of all patches responded to stimulation with ionomycin. Among the records lacking activity of high ionic conductance channel (64%), 15% showed activity of ion channels of low conductance that were blocked by BaCl\(_2\) (data not shown).

Figure 2B illustrates the single channel current-voltage relationship for the pore formation in cell attached after 10 \(\mu\)M ionomycin stimulation. Data were normalized with linear regression producing a slope conductance of 421 ± 4 pS (Table 1) with a reversal potential of 0.2 mV to 150 mM KCl in the pipette. In the absence of ionomycin, neither pore formation nor the channel of low amplitude was observed. In the presence of ionomycin, pore openings showed a conductance level of 407 pS (about 24 pA, Fig. 2C). For the cells stimulated with 10 \(\mu\)M ionomycin, \(P_o\) was 0.47 ± 0.031 (Table 1 and Fig. 2D). The recordings suggested no difference in the mean open time of the open pore in the voltage range tested (\(n = 7, P = 0.018\)). \(P_o\) values were dependent on agonist concentration (Fig. 2D).

**Ionomycin Induced Fluorescent Dye Uptake**

As illustrated in Fig. 3, the time correspondent to 50% of the maximal fluorescent intensity (\(t_{1/2}\)) was not different between EB (\(t_{1/2} = 118 \pm 17\) s) and LY (\(t_{1/2} = 122 \pm 11\) s) on either 2BH4 cells (\(n = 10, P = 0.24\); Table 1) or peritoneal macrophages (\(t_{1/2} = 115 \pm 20\) s; 121 ± 16 s) (data not shown). A dose-dependent relationship between ionomycin concentration and EB uptake is shown in 2BH4 cells (Fig. 3), in peritoneal macrophages (data not shown), or in HEK-293 (cell type that do not express the P2X\(_7\) receptor; data not shown). As a control, we preincubated 2BH4 cells with Ca\(^{2+}\)-free extracellular solution containing EB, and no change in fluorescence occurred (data not shown). We only observed significant EB uptake with a higher concentration (3 \(\mu\)M) of ionomycin (EC\(_{50}\) = 4.69 \(\mu\)M, Fig. 3, A–E and F). The conductance for pore formation did not change in concentrations between 5 and 10 \(\mu\)M ionomycin, reaching a constant value of 410 pS (data not shown), whereas most of the cells died at concentrations higher than 10 \(\mu\)M ionomycin.

The possibility of an increase in the passive intracellular Ca\(^{2+}\) caused by any source such as patch pipette was excluded. Addition of thapsigargin (1–5 \(\mu\)M) showed electrophysiological recordings and dye uptake responses similar to ones induced by ionomycin (supplementary Fig. 1). The dye uptake kinetics and the unitary conductance recorded in the experiments of cell attached for thapsigargin were also similar to those observed by stimulation with ionomycin (data not shown).

To rule out the possible effects of unnoticed ATP release in the patch induced for mechanical stimulation (49, 56, 58, 84), the ectonucleotidase apyrase (2 U/ml) was used in the pipette solution (plus LY) or in the bath (plus EB) when the cell membrane outside the patch was stimulated with 10 \(\mu\)M ionomycin. There was no change attributable to apyrase in the single current recordings or in dye uptake in this condition (data not shown). Thus it is unlikely that ATP leakages or ATP released from other sources contribute to ionophore effects.

Since ionomycin forms a pore with large conductance, we evaluated the molecular size cutoff of this pore. 2BH4 cells treated with ionomycin were permeable to EB, LY, and PI but not to trypan blue or TRITC-dextran (3,000 Da).

**Ionomycin Effect is Dependent on Intracellular Ca\(^{2+}\) Concentration**

To investigate whether the ionomycin effect observed was mediated by intracellular Ca\(^{2+}\), alteration of the intracellular Ca\(^{2+}\) concentration was analyzed on pore formation using the whole cell configuration. Different buffered free Ca\(^{2+}\) concentrations were used in the micropipette solution (solutions F–I; see MATERIALS AND METHODS), as shown in Fig. 3G for dose-response relationship between intrapipette Ca\(^{2+}\) and permeabilization is clear. At low intracellular Ca\(^{2+}\) concentrations, there was a strong inhibition of dye uptake and single currents (data not shown). These experiments were repeated in HEK-293 and IT45-RI cells (do not express the P2X\(_7\) receptor), and such effects were also observed with the same magnitude (data not shown). The \(t_{1/2}\) for ethidium uptake in different intracellular Ca\(^{2+}\) levels increased when intracellular Ca\(^{2+}\) concentration was reduced. The currents passed from macroscopic to microscopic (i.e., to single channel) as the Ca\(^{2+}\) concentration decreased in the pipette solution. Single channel currents lasted for milliseconds when recorded in low Ca\(^{2+}\) concentration, whereas those recorded in 1 mM Ca\(^{2+}\) concentration lasted for seconds (data not shown). These results suggest a possible role for Ca\(^{2+}\) as the intracellular signal to induce a pore in the plasma membrane.

To corroborate the intracellular Ca\(^{2+}\) involvement with the ionomycin-induced pore formation, 2BH4 cells were preincubated with 1,2-bis(o-aminophenoxy)ethane-N\(_2\)N\(_3\)N\(_4\)N\(_5\)-tetraacetic
acid incorporated as its acetoxymethyl ester (BAPTA-AM, a calcium quelator for 15–30 min before ionomycin application).

In the Fig. 4 A and supplementary material Fig. 2, the intracellular Ca\(^{2+}\)/H11001 chelation resulted in inhibition of ionomycin-induced pore formation in a dose-dependent manner.

Signal Transduction Pathway Associated to Ionomycin-Induced Pore Formation

Calmodulin blockers inhibited ionomycin-induced pore formation. As several effects of Ca\(^{2+}\) signaling are mediated by calmodulin, we investigated its role on the ionomycin-induced pore formation. Preincubation (5 to 10 min) of calmodulin antagonists completely inhibited dye uptake and single-channel activity induced by ionomycin in 2BH4 cells (Fig. 4, B and G). Moreover, a dose-response effect was observed between antagonist concentrations and fluorescence intensity after ionomycin application (Fig. 4G). The IC\(_{50}\) for calmidazolium, trifluoperazine, and KN-62 were 1.55 \(\mu\)M, 2.14 \(\mu\)M, and 4.23 nM, respectively (Table 2).

Phospholipase C inhibitors blocked ionomycin-induced pore formation. To test whether the pore induced by ionomycin was dependent on phospholipase C (PLC), the selective blocker U73122 was applied with ionomycin on 2BH4 cells. As shown in Fig. 4, B and G, there was no EB uptake or single channel currents. On the other hand, addition of U73433, an inactive analogue of U73122, promoted EB uptake and single channel currents induced by ionomycin (Fig. 4H).

cAMP-activated kinases are not involved in the ionomycin-induced pore formation. The involvement of cAMP in the ionomycin-induced pore formation was evaluated by applying 300 \(\mu\)M forskolin, which activates adenylate cyclase. Forskolin had no effect on the pore induced by ionomycin (data not shown). Addition of a cAMP-permeable compound, or cAMP itself, also had no effect on the pore induced by ionomycin (data not shown).

Protein kinase C (PKC) is not involved in the ionomycin-induced pore formation. As some PKC isoforms are Ca\(^{2+}\) dependent, PKC inhibitors were used to assess the effects on...
ionomycin-induced pore formation. Preincubation of the cells with the antagonists staurosporine, calphostin C, and Ro-31-8220 before ionomycin application did not block EB uptake and single channel currents (Fig. 4, F and H and Table 2). In addition, PMA, a potent activator of PKC, had no effect on the EB uptake and single channel currents (data not shown).

**Inhibition of ionomycin-induced pore formation with MAPK inhibitors.** 2BH4 cells pretreated with the p38 mitogen-activated protein kinase (MAPK) inhibitor SB-203580 (17) inhibited EB uptake and single channel currents (Fig. 4E). On the other hand, PD-98059, a MEKK inhibitor (57) demonstrated a weak inhibition on the ionomycin-induced pore formation (IC<sub>50</sub> = &gt;150 μM, Fig. 4D) compared with the p38 inhibitor (Table 2 and Fig. 4F).

**Ionomycin induces pore formation analyzed by flow cytometry.** Flow cytometry was used to confirm data obtained with cell-attached patch clamp. There is no cell permeabilization when agonists are not applied in the peritoneal macrophages (negative control; Fig. 5A). Addition of 5 mM ATP induces a subsequent permeabilization (positive control; Fig. 5A2). The rise in the permeabilization observed by ionomycin is dose dependent (Fig. 5, A3, A4, and B). Moreover, intracellular signaling pathways associated to the pore activated by elevation of intracellular Ca<sup>2+</sup> concentration are in agreement with the patch-clamp results (compare Fig. 5 and Table 3 with the Fig. 5C).

SYTO 13 (a dye shown to discriminate apoptotic, live, and dead cells) was used to observe whether the cell population permeabilized by ionomycin would be viable, as indicated elsewhere (32, 78). PI and SYTO 13 were used at the same time, as SYTO 13 modified the peritoneal macrophage morphology after 2 min of incubation. In the Fig. 5, D1 and D2, there are representative negative control experiments (no agonist application). In these conditions, there was no PI uptake (Fig. 5D1). The rise of basal fluorescence of PI seen is due to a SYTO 13 artifact, which was not compensated in the FL-2.
channel. In relation to SYTO 13, an unique peak related to viable cells is observed (Fig. 5D). When cells were treated with 5 mM ATP (positive control), PI fluorescence increased as observed by several groups (Fig. 5D). The SYTO 13 fluorescence decreased (it was split into two peaks; Fig. 5D). The first peak represents live cells and the second peak represents apoptotic cells (early phase). The number of viable cells in the R1 region that were SYTO 13 and PI positive (Fig. 5D) was 71% while 29% of the cells were apoptotic (Fig. 5D). Comparison of this result with the negative control shows that 99.8% of these cells are viable in the R1 region. Addition of 2.5 μM ionomycin induces PI uptake and a decrease of SYTO 13 fluorescence similar to the one observed by ATP application (Fig. 5, D3 and D4). The number of viable cells in the R1 region that uptook the SYTO 13 and PI (Fig. 5D4; first peak) was 63% while 37% of the cells were apoptotic. These results indicate that the majority of cells permeabilized by ionomycin application are viable.

Hemichannel blockers did not inhibit pore formation. Hemichannels formed by connexins or pannexins possess permeability to molecules of up to 1,000 Da (10, 22, 23, 63, 70). We tested whether the ionomycin-induced pore formation might be due to hemichannels activation. Then we added carbenoxolone, 18α-glycyrrhetinic or heptanol (known connexin hemichannel blockers), or carbenoxolone (in concentrations up to 100 μM) and flufenamic acid (known pannexin hemichannel blockers) on 2BH4 cells before stimulation with 10 μM ionomycin. As observed in Fig. 6, B and D, there was dye uptake and single channel currents similar to the control conditions for all hemichannel antagonists tested. These data indicate that hemichannels are not involved with ionomycin-induced pore formation.

Ionomycin-induced pore is inhibited by cytoskeleton blockers. The formation of ionomycin-activated pore might depend on cytoskeleton rearrangements. To test this hypothesis, we used cytoskeleton blockers in 2BH4. Cells preincubated with cytochalasin B (5 min), cytochalasin D (5 min), or colchicine (10 min) showed complete inhibition of dye uptake and single channel currents (Fig. 6A and Table 3). Of note, cytochalasin B (IC50 = 6.3 μM) have presented lower potencies than cytochalasin D (IC50 = 0.33 μM) and colchicine (IC50 = 2.6 μM) (Fig. 6C).

Intracellular Ca2+ rise induces pore formation on LDH release. We ruled out the possibility of necrosis or osmotic lysis induced by addition of the ionophores assayed by the levels of LDH release (19). LDH release from peritoneal macrophage cells were undetectable in the absence of ionomycin (data not shown). LDH release was ≥10% when 2BH4 or macrophages were incubated with 10 μM ionomycin for 1 h (Fig. 6E). Thus, although cell lysis is observed after ionomycin application, it occurs much more slowly and to a lesser extent compared with the rise of intracellular Ca2+-induced pore observed to Ca2+, EB, LY, and PI. In this context, cell lysis was ruled out upon addition of (5 or 10 μM) ionomycin as we measured the LDH released over time. Accordingly, we observed that 10 μM ionomycin increased by 5%, the amount of LDH after 10 min of treatment. In addition, in the window of time that we used for the analysis of fluorescence (5 min), we only found around 2% for the release of LDH on macrophages. These data indicate that the experiments were performed in a period where cellular injury is not present upon ionomycin activation.

Comparison of P2X7 and ionomycin pores in macrophages and HEK-293 cells. Altogether, our data indicate that the pore formed by ionomycin has conductance, permeability, and cutoff similar to a pore induced by ATP (16, 28). To test whether ionomycin-induced pore is the same pore formed by P2X7 receptor, ionomycin was applied in the presence of different blockers of the P2X7 receptor. In the presence of oxidized ATP (1 mM ATP) and/or suramine, two nonselective P2 receptor antagonists, were used to block the P2X7 receptor (12, 37, 42, 79, 81). In addition, TNP-ATP, a selective P2X1 and P2X3 antagonist, was used to block the P2X7 receptor activity (12, 27, 73). None of these inhibitors were able to block the ionomycin-induced pore, suggesting that ionomycin activates a pore distinct of P2X7 receptor or other pore related to P2 receptor family (Fig. 7F; data not shown; respectively).

To further test the participation of P2X7 receptor, we employed IT45-R1 and HEK-293 cells, which do not express P2X7 receptor (7, 64). Addition of ionomycin or TG induced dye uptake and single channel currents (Fig. 7, C, E, and G). In contrast, no pore formation was observed when 1 mM ATP was applied on IT45-R1 cells (Fig. 5, B and G) or on HEK-293 cells (Fig. 5, D and G). Dye-uptake kinetics and conductance values of Ca2+-induced pore were similar to those obtained in 2BH4 cells. Moreover, the ionomycin-induced pore in IT45-R1 and HEK-293 cells showed amplitude (20 ± 3 pA) with a holding value of −60 mV, P0, t1/2 (Table 1), and frequency

### Table 2. Intracellular signaling pathway of ATP- and ionomycin-induced pores

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<tr>
<th>Pore Blockers</th>
<th>2BH4, μM</th>
<th>HEK-293, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 mM ATP</td>
<td>10 μM Ion</td>
</tr>
<tr>
<td>Oxidized ATP (IC50)</td>
<td>125.7     &gt; 500</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>Calmidazolium (IC50)</td>
<td>&gt; 100     1.56</td>
<td>4.8</td>
</tr>
<tr>
<td>TFP (IC50)</td>
<td>&gt; 100     2.14</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>SB203580 (IC50)</td>
<td>0.0336    37.6</td>
<td>44.7</td>
</tr>
<tr>
<td>PD98059 (IC50)</td>
<td>10.72     87.47</td>
<td>241.45</td>
</tr>
<tr>
<td>Staurosporine (IC50)</td>
<td>&gt; 300     &gt; 300</td>
<td></td>
</tr>
<tr>
<td>Ro-31-8220 (IC50)</td>
<td>1.11      &gt; 300</td>
<td></td>
</tr>
<tr>
<td>U73343 (IC50)</td>
<td>&gt; 300     50</td>
<td></td>
</tr>
<tr>
<td>U73122 (IC50)</td>
<td>&gt; 300     &gt; 50</td>
<td></td>
</tr>
<tr>
<td>BAPTA-AM (30 μM)</td>
<td>Blocked   Blocked</td>
<td></td>
</tr>
<tr>
<td>HMA (IC50)</td>
<td>1.32      7.88</td>
<td>16.36</td>
</tr>
</tbody>
</table>

Summary of experiments performed to assess the effect of blocking of cell-signaling pathways on the pore induced by the increase of intracellular calcium and the pore activated by the ATP. These experiments were done in cells attached at 37°C. Cell types that express (2BH4 cells) or do not express (HEK-293 cells) the P2X7 receptor were used. In the column Pore blockers, various compounds are listed (cited in the text) that were applied to evaluate whether or not they block a pore mentioned above. The two subsequent columns referring to 2BH4 cells show the values of IC50 obtained for each of the blockers for both the pore induced by 1 mM ATP or to ionomycin (Ion). The last two columns are similar to the ones mentioned by previous columns.
[Previously extracted text]

**DISCUSSION**

In this work, we investigated the properties of a pore activated by raising intracellular Ca\(^{2+}\), which shares several characteristics with P2X7-associated pore. However, only the results related to ionomycin are shown as representative since either A23187 or thapsigargin responded similarly to ionomycin.

![Fig. 5. ION-induced pore formation analyzed in flow cytometry.](http://apcell.physiology.org/)

(data not shown) similar to ionomycin-induced pore in 2BH4 cells. Altogether, these data indicate that ionomycin and TG activate a pore without the participation of the P2X7 receptor.

In relation to the intracellular signaling pore activated by the increase of intracellular Ca\(^{2+}\) in HEK-293 cells, it is shown that the signaling pathway activated by ionomycin in cells 2BH4 is similar to the pathway present on HEK-293 cells (Tables 2 and 3). Moreover, this pathway activated by the increase of intracellular Ca\(^{2+}\) has different components compared with the ones activated by ATP. The pore activated by the increase of intracellular Ca\(^{2+}\) needs calmodulin as well as PLC, whereas the pore activated by ATP needs PKC activity.

Other differences between the pore activated by ATP and the pore activated by ionomycin are the former opens only in the range of 30–37°C in the peritoneal macrophages (data not shown), whereas the latter works below 25°C in the same cells (data not shown).

**Table 3. Effects of cytoskeleton, caspase on ATP, and ionomycin-induced pores**

<table>
<thead>
<tr>
<th>Antagonists (IC(_{50}))</th>
<th>ATP (IC(_{50}))</th>
<th>2BH4 (IC(_{50}))</th>
<th>HEK-293 (IC(_{50}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochalasin B</td>
<td>5.73 μM (n = 12)</td>
<td>7.2 μM (n = 11)</td>
<td>7 μM (n = 6)</td>
</tr>
<tr>
<td>Cytochalasin D</td>
<td>144 nM (n = 11)</td>
<td>700 nM (n = 9)</td>
<td>1.2 μM (n = 6)</td>
</tr>
<tr>
<td>Colchicine</td>
<td>1.14 μM (n = 11)</td>
<td>3 μM (n = 9)</td>
<td>4.1 μM (n = 6)</td>
</tr>
</tbody>
</table>

Summarized data on the modulation of cytoskeleton on the increase of intracellular calcium and on the properties of the pore activated by the ATP. These experiments were done in cell attached to 37°C. In the column “antagonists”, cytoskeleton blockers are listed. The “ATP” column represents the values of IC\(_{50}\) for cytoskeleton blockers in 2BH4 cells stimulated with 1 mM ATP. The rear columns represent the values of IC\(_{50}\) for 2BH4 and HEK-293 cells stimulated with 10 μM ionomycin.
Because many properties of the pore activated by the rise in intracellular Ca\textsuperscript{2+} are similar to P2X\textsubscript{7} receptor pore, we tested whether P2X\textsubscript{7} receptor antagonists could block the pore activated by Ca\textsuperscript{2+}. We found that this pore is not blocked by P2X\textsubscript{7} receptor antagonists (Fig. 7F). Furthermore, we showed that HEK 293 and IT45 R1, which do not express P2X\textsubscript{7} receptor, have the pore activated by intracellular Ca\textsuperscript{2+} (Fig. 7G). In line with these data, two different groups showed that the pore activated by maitotoxin, which is also Ca\textsuperscript{2+} dependent, was not blocked by P2X\textsubscript{7} receptor antagonists in THP-1 and CHO (K1 strain) cells (20, 50).

The increase in intracellular Ca\textsuperscript{2+} concentration is a factor that is related to the mechanism of opening of several pores, either acting directly or indirectly. In this context, we listed some indirect factors, mainly related to the mechanism of opening of the pore associated with P2X\textsubscript{7} receptor. Interestingly, several reports described drugs that are able to increase intracellular Ca\textsuperscript{2+} concentration, acting synergistically with ATP and potentializing P2X pore formation. Ladjova and coworkers (36) demonstrated that 4-aminopyridine, a potassium channel blocker, activates elevation of the intracellular Ca\textsuperscript{2+} concentration, which induces a pore of ~350 Da in human T lymphocytes (45). Fernandez and collaborators demonstrated that propofol, which is an intravenous anesthetic agent (68), increased the intracellular Ca\textsuperscript{2+} concentration and potentialized the P2X\textsubscript{4} pore formation in rat submandibular acinar cells (29, 25). Ferrari and colleagues showed that polymixin B, which is a natural peptide used as an antibiotic, potentializes ATP effects in the membrane permeabilization and cytotoxic effects induced by extracellular Ca\textsuperscript{2+} influx mediated by P2X\textsubscript{7} receptor. Recently, Faria and colleagues demonstrated that the ATP-induced pore can be potentialized by Ca\textsuperscript{2+} ionophores.

In addition, Seman and collaborators showed that NAD can activate the P2X\textsubscript{7} receptor by an indirect mechanism that involves NAD acting as substrate for an ecto-ADP-ribosyl-
transferase that covalently binds to ADP-ribosylates a specific arginine residue on the P2X7 extracellular loop. This NAD activation induces intracellular Ca\(^{2+}\) increase, which triggers pore formation and cell death in mouse mature T cells. Elssner and colleagues (25) demonstrated that the peptide LL37 derived from human cathelicidine induces ATP release, pore formation, caspase-1 activation, and IL-1\(^{β}\) release. All these effects were caused by direct P2X7 receptor activation. Taken together, these data suggest that the rise of intracellular Ca\(^{2+}\) is important to pore formation. Moreover, these results indicated the possibility of an unique pore possibly activated by several extracellular signals and all of them with similar intracellular signaling pathways.

Previous reports of pores directly activated by the elevation of intracellular Ca\(^{2+}\) have been described (74, 80, 82). As our data indicate that the pore activated by the rise of intracellular Ca\(^{2+}\) is not the one associated with P2X7 receptor, we investigated whether this pore could be one already described in the literature or a new one. Thus we compared our results for the pore activated by the increase in intracellular Ca\(^{2+}\) with data described for diverse pores with similar properties. Anion channels are a group of channels with few data, so far, at the molecular and biophysical level. Nevertheless, these channels carry out a few functions such as ATP and glutamate release and transport of large anionic molecules. Among the anion channels, there are two types that show properties similar to P2X7 receptor-associated pore. The first one is the maxi anion channel, an ATP (6, 21) and glutamate (47) permeable channel that depends on the increase in intracellular Ca\(^{2+}\) (40, 46) and it possesses a high unitary conductance (6, 21) (\(\sim\)400 pS) (9, 47). Moreover, this channel can be activated by ischemia or hypoxia (21), where raising intracellular Ca\(^{2+}\) is also important (13). We ruled out the participation of maxi anion channel, based on experiments using Ca\(^{2+}\) ionophores in

![Fig. 7. Pore induced by ION is not dependent on P2X7 receptor. Experiments were conducted using cell-attached configuration or whole cell at 37°C. EB was applied in the bath. A: preincubation for 1 h with P2X7 receptor blocker, 300 µM oxidized ATP in the bath before 10 µM ION application. B: addition of 1 mM ATP in the bath of IT45-RI cells; C: addition of 10 µM ION in the bath of IT45-RI cells. D: 1 mM ATP in the bath on HEK-293 cells; E: 10 µM ION application in the bath on HEK-293 cells. Ionic currents (to the right of fluorescence data) elicited by ION application are indicated with vertical arrows. Open arrows to the left of ionic currents indicate \(I = 0\) pA. Holding potential was 60 mV. F: fluorescence intensity was recorded as a function of P2X receptors antagonist concentrations in the bath. G: EB uptake plotted for each curve as fluorescence maximal intensity after agonist application in the 2BH4, IT45-RI, or HEK-293 cells. Basal values were subtracted, and the curves were normalized to maximal fluorescence intensity obtained for 20 µM digitonin application after 5 min for each curve. Values represent means ± SD of 3–6 independent experiments. A: solution A; B: solution B; F: solution F, oxATP, oxidized ATP; TG, thapsigargin.
Inside-out configuration (data not shown). This evidence indicates that the pore formed by raising intracellular Ca\(^{2+}\) depends on cytoplasmic factors, whereas the pores formed by maxi anionic channel do not. Moreover, the maxi anion blocker okadaic acid did not block the elevation of intracellular Ca\(^{2+}\)-induced pore (data not shown).

Another possible anionic channel is voltage-dependent anion channel (VDAC). Bahamonde and Valverde (3) demonstrated that VDAC protein originally discovered in outer mitochondrial membrane is also present in the plasma membrane of the cells and they can be activated by toxic stimulus, an event preceded by caspase activation in neural apoptosis (24). Akanda and Elinder (2) observed that membrane VDAC has a large conductance (400 pS) and occasionally subconductance states of \(\sim 28\) and \(220\) pS. Gincel and collaborators (34) reported that mitochondrial VDAC is permeable to Ca\(^{2+}\) and that Ca\(^{2+}\)-binding sites are involved in the modulation of its channel and of the mitochondrial permeability transition pore. We discarded the possibility of the pore activated by raising intracellular Ca\(^{2+}\) to be a plasma membrane VDAC, since the first one is not voltage dependent. Moreover, the VDAC antagonist 4'-disothioiocyanato-2,2'-disulfonic acid stilbene did not block the opening of the pore activated by the elevation of intracellular Ca\(^{2+}\) (data not shown).

Based on a recent report of connexin hemichannels activation by an increase in intracellular Ca\(^{2+}\) (80), we tested whether the pore described in this work could be a connexin hemichannel. However, addition of hemichannel antagonists on 2BH4 or HEK-293 cells had no effect in the elevation in intracellular Ca\(^{2+}\) promoted by ionomycin (Fig. 6).

Recent data (48, 59) had shown that pannexin 1 is a critical protein in the formation of the pore associated with P2X\(_7\) receptor, independently of P2X\(_7\) receptor. Therefore, experiments were done in the presence of pannexin 1 antagonists as carbeneoxolone and flufenamic acid. We observed that both pannexin antagonists did not inhibit the increase in intracellular Ca\(^{2+}\)-induced pore. However, pannexin 1 blockers did block partially the pore formation associated to P2X\(_7\) receptor (48) based on a residual dye uptake as an alternative unspecified pathway. In addition, Pelegrin and Surprenant (60) had shown that the pore formed by maitotoxin, which is very similar to the P2X\(_7\) pore, was not blocked by pannexin 1 antagonists. This evidence strengthens the possibility that another pore exists or that other pores are involved in the formation of a complex associated to P2X\(_7\) receptor.

Among all these pores cited, the pore activated by maitotoxin is the one that possess a closer profile to the one activated by an elevation of intracellular Ca\(^{2+}\). In addition, this pore depends on the intracellular Ca\(^{2+}\) increase so that its activation occurs (82). This pore does not depend on the P2X\(_7\) receptor to open (50), and its opening does not depend on pannexin 1 (60). Moreover, this pore possesses similar biophysics characteristics such as voltage independence, a nonselective pore, a cut-off around 900 Da (66), and a pore opening with single conductance of 400 pS (85).

In relation to the intracellular signaling pathways associated with this new pore, addition of calmodulin antagonists (KN-62, calmidazolium, and trifluoperazine) before ATP application inhibited the pore activated by raising intracellular Ca\(^{2+}\) (Fig. 4, Table 2). Other groups showed similar results with the pore activated by maitotoxin stimulation (20, 50, 66). These data demonstrate that the pore activated by high intracellular Ca\(^{2+}\) concentrations are susceptible to calmodulin blockers and may share properties with maitotoxin pore.

In addition, U73122, a PLC blocker, inhibited the intracellular Ca\(^{2+}\)-induced pore formation in a concentration-dependent manner (Fig. 4). Estacion and Schiling (26) showed that U73122 blocked maitotoxin-induced cell death. However, this effect was not due to PLC inhibition, because the U73343, which is an ineffective structural analogue of U73122, also blocked maitotoxin-induced cell death.

Moreover, the intracellular Ca\(^{2+}\)-induced pore also depends on MAPK (Fig. 4). Indeed, Malaguti and colleagues (51) demonstrated that ERK1 and ERK2 are involved in cytotoxic responses mediated by maitotoxin. On the other hand, Donnelly-Roberts and colleagues (20) demonstrated that the pore formation induced by maitotoxin was not blocked by MAPK blockers or by caspase-3 antagonist. However, we observed an intracellular Ca\(^{2+}\) raising induced pore formation inhibition when DEVD, a caspase-3 inhibitor, was added (data not shown).

Our data indicate that the pore activated by raising intracellular Ca\(^{2+}\) depends on calmodulin, PLC, and MAPK. This profile is similar to maitotoxin pore intracellular signaling except for the inhibition of MAPKs and caspase-3. The maitotoxin-induced pore and the pore activated by raising of intracellular Ca\(^{2+}\) can be a new pore, but we still cannot.
discard the possibility of a unique pore activated by distinct intracellular pathways. This novel pore might be functional in several circumstances where an appropriate elevation of intracellular Ca\(^{2+}\) is required. In this regard, we tested whether cytoskeleton blockers might inhibit Ca\(^{2+}\)-activated pore. In fact, these modulators inhibited Ca\(^{2+}\)-activated pore and P2X\(_7\) pore (Fig. 6 and Table 3). We observed that ionomycin-induced pore formation on either 2BH4 or HEK-293 cells was blocked by colchine (microtubule inhibitor), cytochalasin B (actin inhibitor), and cytochalasin D (intermediary filaments inhibitor; Fig. 6, Table 3). The pore activated by ATP was also blocked by cytoskeleton antagonists, which suggests that cytoskeleton modification is a common mechanism in the opening of both pores. However, our data do not rule out other possibilities of plasma membrane disruption that allow cytosolic components to escape and reseal preventing the death of wounded cells (52, 75).

Beyond the pore studied in this work, the importance of raising intracellular Ca\(^{2+}\) concentration in the opening of other pores is also highlighted. In this line, addition of the lymphokine leukoregulin induces membrane permeabilization on tumor cells to low-molecular weight fluorescent dyes (4, 5). They also showed that membrane permeabilization was induced by the Ca\(^{2+}\) ionophore A23187 on the same cell type in a calmodulin-dependent manner. This suggests that the extracellular and intracellular Ca\(^{2+}\) is important for the pore formation, and these results are similar to the ones reported here.

In addition, Alonso and Carrasco (1) showed that A23187 induced hygromycin uptake in HeLa cells. De and Friedberg (18) also demonstrated that A23187 induced membrane permeability in mouse fibroblasts. In this paper, the authors showed that 3T6 cells and other transformed lineages induced membrane permeabilization, which was dependent on extracellular and intracellular Ca\(^{2+}\) increase. In addition, Vuyst and colleagues (80) reported that A23187 induces intracellular Ca\(^{2+}\) increase, ATP release, and PI uptake in C6 cells transfected with Cx32. Other ionophores such as amphotericin B and nigericin were also reported to permeabilize mammalian cells to macromolecules; however, these ionophores form its pores independently of cytoplasmic factors. Another recent data reported that palytoxin, a Na\(^{+}\)-K\(^{-}\)-ATPase-activating toxin, induces uptake of EB, but not of YOPRO-1, by bovine aortic endothelial cells with subsequent cell death. This effect was blocked by ouabain, a Na\(^{+}\)-K\(^{-}\)-ATPase inhibitor. Therefore, membrane permeabilization induced by palytoxin occurred after the increase of intracellular Ca\(^{2+}\) concentration (65). Several other molecules such as epidermal growth factor (EGF) (55, 86) or nonsteroidal anti-inflammatory drugs (NSAIDs) are also known to increase intracellular Ca\(^{2+}\) as well as membrane permeabilization (77), leading to apoptosis (35). NSAIDs were able to raise intracellular Ca\(^{2+}\) and permeabilize the cell membrane to calcine (77).

Taking together all these data, we suggest in this work a “new” pore activated by raising of intracellular Ca\(^{2+}\) (Fig. 8A). We believe that this pore is part of a complex of membrane pores that can be activated by different extracellular signals, increasing the intracellular Ca\(^{2+}\) concentration, and regulating distinct intracellular signaling pathways (Fig. 8A). We do not exclude the involvement of other possibilities related to the opening of these pores, such as the pore activated by maitotoxin and the one described here are the same entity, since J both have similar pharmacological and biophysical properties, 2) they are not blocked by panexin-1 antagonists (Fig. 8B; 48, 60), and 3) possibility that all three pores are the same entity activated by different intra- or extracellular signaling pathways (Fig. 8C).

To our knowledge, we demonstrated for the first time, using a combination of electrophysiological and dye uptake techniques, that raising intracellular Ca\(^{2+}\) is able to activate a pore similar to the large pore associated with P2X\(_7\) receptor. In addition, this pore activated by Ca\(^{2+}\) might be a new form of introducing hydrophilic drugs inside cells. The potential therapeutic application is currently under investigation in our laboratory.

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REFERENCES


A PORE INDUCED BY RISING INTRACELLULAR Ca2+


