P2X7 purinoceptor expression in *Xenopus* oocytes is not sufficient to produce a pore-forming P2Z-like phenotype

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Abstract The purinergic rP2X7 receptor expressed in a number of heterologous systems not only functions as a cation channel but also gives rise to a P2Z-like response, i.e. a reversible membrane permeabilization that allows the passage of molecules with molecular masses of ≥300 Da. We investigated the properties of rP2X7 receptors expressed in *Xenopus* oocytes. In two-electrode voltage-clamp experiments, ATP or BzATP caused inward currents that were abolished or greatly diminished when $NMDG^+$ or $choline^+$ replaced Na^+ as the principal external cation. In fluorescent dye experiments, BzATP application did not result in entry of the fluorophore YO-PRO-1^{2+}. Thus, rP2X7 expression in *Xenopus* oocytes does not by itself give rise to the pore-forming P2Z phenotype, suggesting that ancillary factors are involved.

Key words: P2 purinoceptor; Pore formation; Electrophysiology; Oocyte expression

1. Introduction

ATP can serve as an extracellular ligand for three classes of receptors which are designated P2X, P2Y and P2Z. P2Y receptors are G-protein-coupled membrane proteins; P2X receptors are ion channels that are permeable to cations; and P2Z receptors, when activated, permeabilize cells to molecules as large as 900 Da [1]. Thus, activation of P2Z receptors appears to result in the generation of large pores in the plasma membrane. P2Z receptors have attracted considerable attention since they are expressed in macrophages and lymphocytes where they may play a role in signaling between cells or in the interaction of these immune cells with their targets [2].

Recently evidence has been offered that the P2Z receptor is actually one member of the P2X class, seven different members of which have now been cloned [2,3]. The most recently cloned of these, rP2X7 (P2X7 from the rat *Rattus norvegicus*) functions as a cation channel at normal concentrations of divalent cations. But its activation by ATP or BzATP in the presence of submillimolar concentrations of external divalent cations results in permeability for larger molecules like $NMDG^+$ (195 Da) or the dye YO-PRO-1^{2+} (376 Da). This pore forming effect was demonstrated in a variety of cultured cells transfected with the rP2X7 receptor [3].

To investigate in more detail the ability of rP2X7 receptors to induce permeability to larger molecules, we decided to use the *Xenopus* oocyte expression system. We did so because of earlier studies on mouse macrophages which have been shown to have a P2Z-like pore-forming response. When poly(A)$^+$ RNA from mouse macrophages is injected into *Xenopus* oocytes, ATP application in the presence of submillimolar concentrations of external divalent cations causes the oocytes to become permeable to $NMDG^+$ and large molecular weight dyes [4]. We decided to repeat these observations by injecting rP2X7 cRNA into the oocytes. Unexpectedly, ATP or BzATP application to these cells did not result in permeabilization of the cells to large molecular weight compounds. This conclusion was reached on the basis of two separate lines of experimentation, electrophysiological monitoring of ionic currents under voltage-clamp and fluorescence measurements of dye entry. Thus it would appear that ATP-induced permeabilization (i.e. pore formation) depends on factors in addition to the rP2X7 receptor itself.

2. Materials and methods

2.1. In vitro transcription and plasmid preparation

Plasmid cDNA encoding the rat rP2X7 receptor (GenBank accession number X95882) was kindly supplied by Dr. Gary Buell (Glaxo Institute for Molecular Biology, Switzerland). Plasmids were prepared by standard procedures and purified using a Plasmid Maxi kit (Qiagen Inc. Chatsworth, CA). Restriction mapping and partial sequencing were performed to verify that the cDNA in our plasmid was that of the sequence published for rP2X7 receptor. Plasmids were linearized in preparation for transcription run-off using the *Xho*I restriction site. In vitro transcription was performed using a mMessage mMachine kit (Ambion Inc. Austin, TX). The only departure from the manufacturer's protocol was that, following DNAase treatment, the full-length, capped cRNA was purified using Chroma Spin 100 columns equilibrated with DEPC-treated water (Clontech, Palo Alto, CA). cRNA samples were run on a denaturing agarose RNA gel and were used only if they were of the expected size and ran as a single band.

2.2. Oocyte preparation and cRNA injection

Oocytes were isolated from *Xenopus laevis* (supplied by Nasco, Fort Atkinison, WI, and Xenopus 1, Dexter, MI) using standard protocols [5] and scored according to Dumont [6]. Only stage V or VI defolliculated oocytes were used. cRNA was used at a 1 μg/ml concentration in DEPC-treated water and 25–50 ng injected into each oocyte using a Drummond 'Nanoject' motorized microinjection device (Drummond Scientific Co., Broomal, PA) Sham-injected oocytes were injected with the appropriate amount of DEPC-treated water alone. Oocytes were then incubated in Barth's solution with gentamicin (50 mg/ml; Sigma) and penicillin streptomycin (100 U/ml; Sigma) at 18°C and used from 2 to 5 days following injection. To diminish the effects of Ca^{2+}-activated Cl^- currents endogenous to *Xenopus* oocytes, the oocytes were injected with 40–50 nl of a 50 mM EGTA or BAPTA solution (final concentration ~1 mM) into oocytes 30 min prior to experimentation. Such protocols have been used in the past to successfully inhibit these contaminating currents [7]. In some experiments no BAPTA or EGTA

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Abbreviations: BzATP, 2′- and 3′-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate; NMDG+, *N*-methyl-D-glucamine; rP2X7, rat P2X7

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injection was carried out. All experiments were performed at room temperature (20–23°C).

2.3. Electrophysiology

Standard two-electrode voltage clamp techniques [8] were used to investigate rP2X7 receptor responses in oocytes. A Dagan TEV-200 two-electrode voltage-clamp amplifier and pClamp 6 software were used for control of experimental protocols and data conditioning and acquisition. For most experiments oocytes were held at a membrane potential of −50 mV. Voltage ramps were used to investigate current–voltage relationships. The potential was first moved from the holding level of −50 to −80 mV then moved gradually to +20 mV (voltage ramp rate 12.5 mV/s) and finally back to the holding level. When leak subtraction was employed, currents obtained by ramps performed under control conditions were subtracted from currents under test conditions. Microelectrodes were filled with a 3 M KCl solution filtered with a sterile Millex-GV 0.22 μm filter unit (Millipore Bedford, MA). Electrode resistance for the voltage electrode ranged from 2–5 MΩ, and for the current passing electrode resistance was typically 1 MΩ. Concentration–response curves were fit to logistic functions using a Marquardt-Levenberg algorithm as implemented in a commercial software package (SigmaPlot; Jandel Corporation, CA)

2.4. Solutions and drugs

The flow of the extracellular bathing solution was controlled using a DAD-12 computerized perfusion device (Adams and List, NY) coupled to a multipath manifold located at the entry portal to the bath chamber. This arrangement enabled fairly rapid changes in bathing solution in the 150 μl chamber. Five to 10 s were required to completely exchange the bathing solution in the chamber. Thus, the solution surrounding the oocyte which was located at the mouth of the manifold outlet was exchanged even more rapidly. For most experiments a bathing solution low in divalent cation concentration was used which contained (in mM) Na^+ 96, K^+ 2, Cl^- 98, HEPES 5, Ca^{2+} 0.1, pH 7.5; Ba^{2+} at the same concentration (0.1) was sometimes substituted for Ca^{2+}. In BAPTA- or EGTA-injected oocytes we could see no difference between recordings made in the presence of Ba^{2+} or Ca^{2+}. In some experiments BAPTA or EGTA injection was not carried out, but the general conclusions were the same in all cases. ATP or BzATP were obtained from Sigma Chemicals (St. Louis, MO) and were dissolved at working concentrations directly into bathing solu-

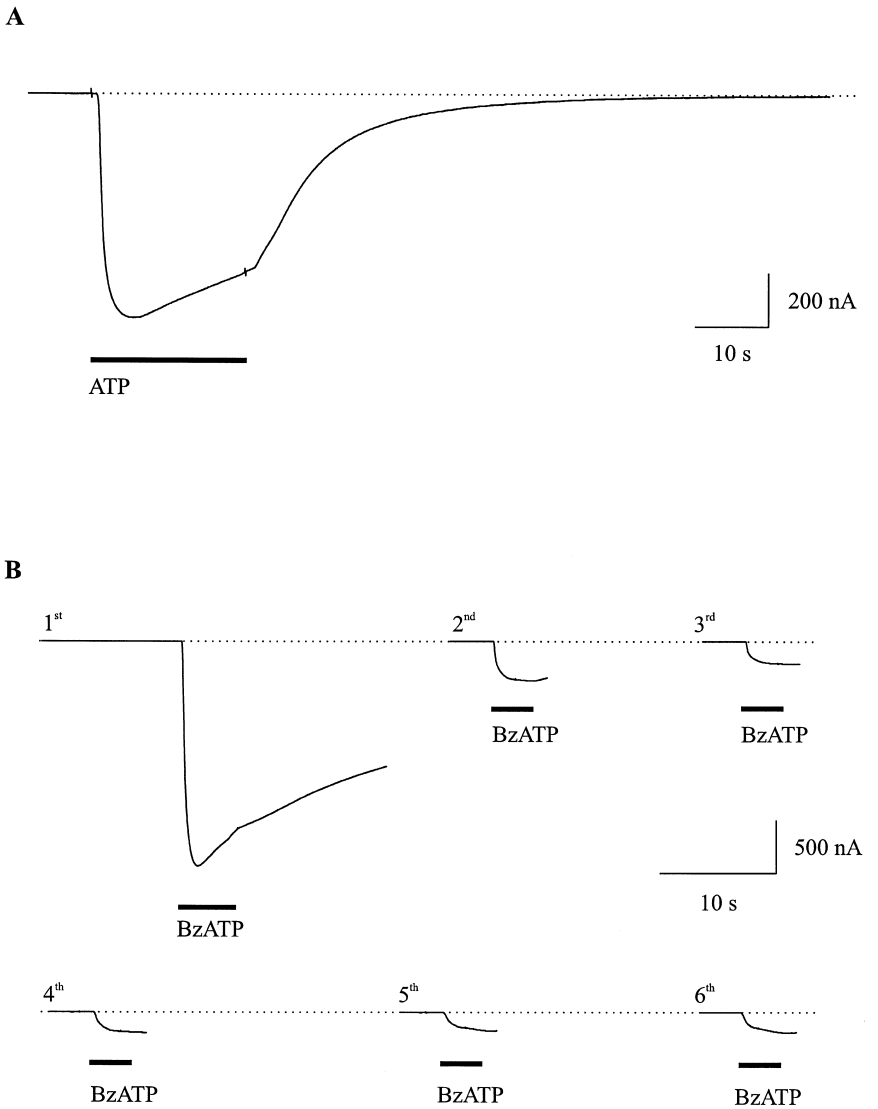

Fig. 1. Comparison of ATP and BzATP activated currents. A: Application of ATP (300 μM; calculated free ATP^{4-}, 118 μM) to a BAPTA-treated oocyte injected with rP2X7 cRNA reversibly activated a large inward current. B: In a different oocyte, application of BzATP (100 μM; calculated free $BzATP^{4-}$, 35 μM) also activated an inward current. The first application of BzATP resulted in the largest current which is shown in the trace at upper left. Smaller currents elicited by subsequent applications of BzATP at 12 min intervals are shown in the remaining traces. Note that the scale is identical for the first trace and the subsequent traces which depict much smaller currents. The period of ATP or BzATP application is shown by the horizontal bar beneath the current trace. Holding potential was −50 mV in each case.

tion. ATP or BzATP were used at total final concentrations of 300 μM or 100 μM which gave calculated free concentrations of 118 μM and 35 μM, respectively, in a 100 μM Ca^{2+} bathing solution [9]. (Unless otherwise specified, total concentration is given in the text.) In making calculations for free [$BzATP^{4-}$], the same dissociation constants were used as for ATP. Solutions containing NMDG (MW 196; Fluka, Ronkonkoma, NY) or choline chloride (MW 140; Sigma), were made by substituting these large organic cations for Na^+ and K^+. *Staphylococcal* alpha-toxin was obtained from Life Technologies (Grand Island, NY) and Saponin from ICN Pharmaceuticals (Costa Mesa, CA).

2.5. YO-PRO-1 entry

YO-PRO-1 (MW of the di-iodide salt=629; 1 mM stock in DMSO; Molecular Probes, Eugene, OR) was dissolved into 10 ml of 0.1 mM Ca^{2+} bathing solution and divided equally into two separate 35 mm plastic petri dishes to give a final YO-PRO-1^{2+} concentration of 10 μM. To one of these dishes BzATP was added to a give a final total concentration of 100 μM. Oocytes were placed in either dish for 5 min and then returned to a dish containing 0.1 mM Ca^{2+} bath alone. Fluorescence intensity of oocytes was monitored using a Nikon Diaphot fluorescence microscope equipped with a fluorescein isothiocyanate filter set, a 20× objective and a digital camera (Photometrics, Tuscon, AZ). 100 ms exposures were taken and stored on computer disk for later analysis using custom software to define and measure the average intensity of a 10×10 pixel region of interest. Fluorescence intensity was plotted as the average number of photons detected in this region during the 100 ms exposure and was designated as counts/100 ms. Background fluorescence intensity was determined by making measurements of oocytes that had never been incubated in YO-PRO-1^{2+}. To ensure that oocytes used for these measurements were indeed expressing functional rP2X7 receptors, we randomly chose five oocytes from each batch and tested for appearance of BzATP-activated currents using two electrode voltage clamp. For each batch tested, five of five oocytes showed BzATP-activated currents, suggesting that expression efficiency was close to 100%. This was also our experience with the oocytes during the electrophysiological experiments described above.

In experiments utilizing HEK293 cells we assayed YO-PRO-1^{2+} uptake in cells cultured onto 12×12 mm glass coverslips. YO-PRO-1 fluorescence intensity was measured using a Nikon Diaphot microscope equipped with a 40× objective; both a brightfield and a fluorescent image of the same microscope field were stored onto computer disk for later analysis. To determine the percentage of cells that were YO-PRO-1^{2+} positive, the total number of cells in the brightfield image was counted manually. The number of cells that were fluorescent, indicating YO-PRO-1^{2+} entry, was then determined from the corresponding fluorescent image.

3. Results

3.1. ATP or BzATP activates cationic currents in Xenopus oocytes injected with cRNA encoding the rP2X7 receptor

Xenopus oocytes were either injected with cRNA for the rP2X7 receptor or sham-injected with a similar volume of DEPC-treated water. Application of ATP (300 μM; calculated free ATP^{4-}, 118 μM) or BzATP (100 μM; calculated free $BzATP^{4-}$, 35 μM) in solutions containing a low concentration of divalent cations (100 μM Ca^{2+} or Ba^{2+}) produced no response in sham-injected oocytes ($n=6$) but yielded large currents in oocytes injected with rP2X7 cRNA (Fig. 1.) The currents reversed direction at a membrane potential of −10 to −20 mV ($n=12$), as expected for the cationic currents carried by P2X channels.

When ATP was used, the current trace took about a minute to return to baseline (Fig. 1A) upon termination of ATP application. Repeated applications of ATP produced currents of identical amplitude, given a sufficiently long interval (20 min or more) between applications.

When BzATP was applied (Fig. 1B), the resulting currents were typically larger and desensitized more quickly than those generated in response to ATP. Recovery also took longer, the current trace often requiring 10 min or more to return to baseline upon termination of BzATP application ($n=9$). (Slow recovery of BzATP-activated current was also observed in earlier work on HEK293 cells transfected with rP2X7 cDNA [3].) In most cases, repeated applications of BzATP (1–30 s; 100 μM) at 10–12 min intervals produced currents that were far smaller than the initial response (Fig. 1B), most likely due to a long-lasting desensitization. Even when periods

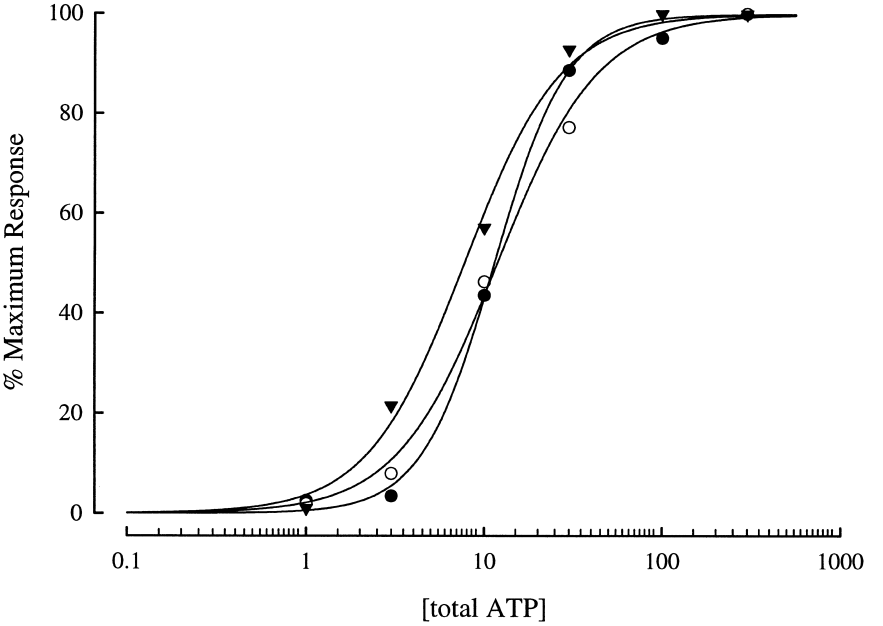

Fig. 2. ATP concentration–response relationship in oocytes expressing rP2X7 receptors. Peak inward current was measured in response to applications of ATP in three different BAPTA-injected oocytes. Data for each of the oocytes is shown fit with a logistic curve constrained to 0 and 100% at, respectively, the minimum and maximum response. Averaged, normalized values were also fit with a single logistic curve, yielding an EC_{50} of 10 μM (3.3 μM for calculated free ATP^{4-}) and a Hill coefficient of 1.7. Holding potential was −50 mV.

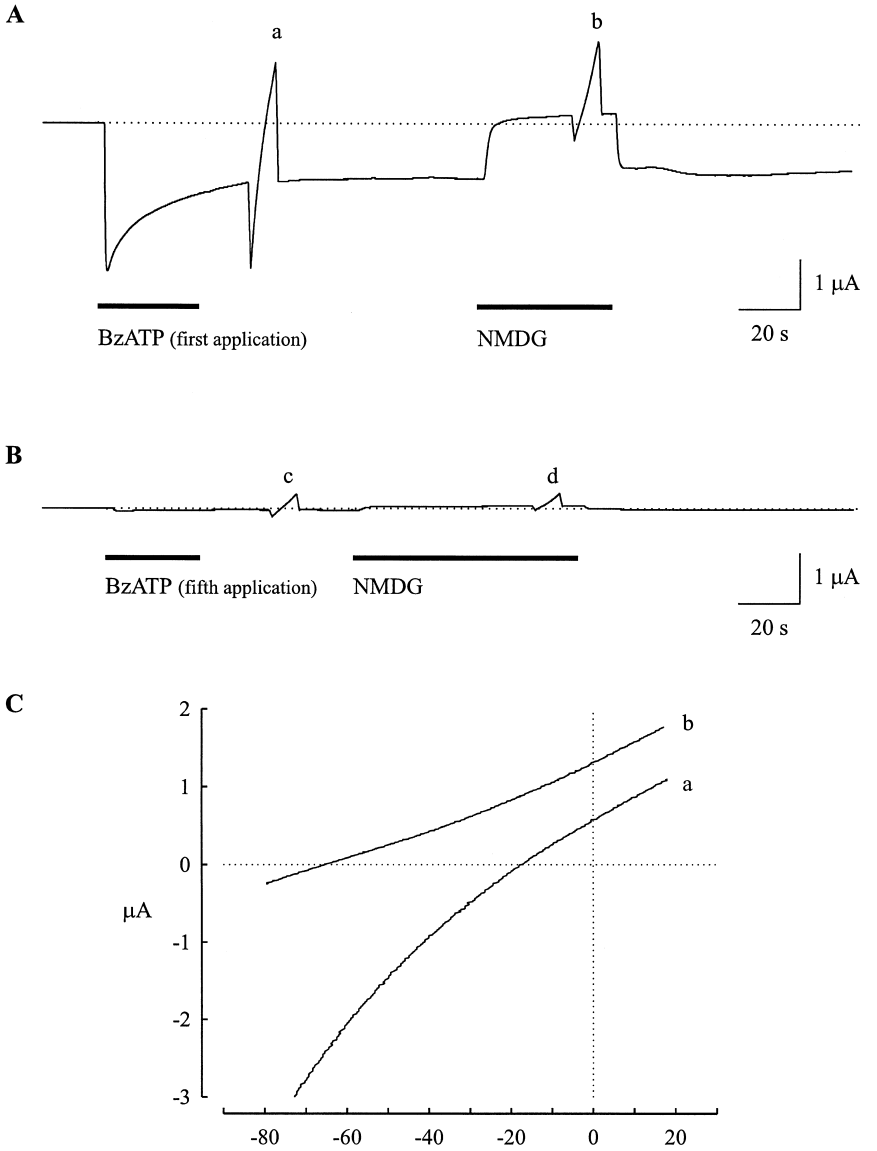

Fig. 3. BzATP-activated inward currents are markedly decreased when $NMDG^+$ is substituted for extracellular Na^+. A: Current trace showing result of first application of BzATP to an oocyte injected with rP2X7 cRNA. BzATP application indicated by the bar below the trace lasted for 30 s. (At 'a' and 'b' a voltage ramp was applied, i.e. the voltage was changed from the holding level of −50 to −80 mV, then moved gradually to +20 mV at a rate of 12.5 mV/s and then back to the holding potential.) The oocyte was continuously superfused, so that BzATP was washed away within a few seconds after application was terminated. When $NMDG^+$-containing superfusate was substituted for the normal Na^+-containing superfusate, the BzATP-activated inward current was eliminated; upon restoration of normal superfusate, the BzATP-activated inward current returned. B: BzATP was then applied at 12 min intervals after complete recovery from the BzATP-induced current shown in (A). The fifth such application is shown here, the protocol being the same as in (A) with the same sort of voltage ramps applied at 'c' and 'd'. The period of BzATP or NMDG application is shown by the horizontal bar beneath the current trace. C: Current–voltage curves obtained from voltage ramps applied at 'a' and 'b' in (A). When $NMDG^+$ replaced Na^+ in the external solution, the reversal potential shifted from ∼−15 mV to ∼−65 mV.

of 1 h were allowed between applications of BzATP, full recovery did not always occur. However, in some cases repeated applications of BzATP produced an increasing current which desensitized very gradually (see below, Fig. 4A).

The concentration–response relationship for the rP2X7 receptors expressed in *Xenopus* oocytes was determined using ATP (Fig. 2). Several measures were taken to avoid possible distortions that might arise as a result of desensitization in response to application of ATP. First, a minimum of 20 min was allowed to elapse between successive applications of ATP to permit recovery from desensitization. Second, for each oocyte tested, the application of at least one concentration of ATP was repeated during the course of the experiment to ensure that responses to that concentration were unchanged over time. The EC_{50} derived from these relationships was around 10 μM, almost an order of magnitude less than that observed previously in HEK293 cells transfected with rP2X7 cDNA [3]. In terms of free ATP^{4-} which is thought to be the ligand for P2X7 and P2Z receptors (but see Ugur et al. [10]), the EC_{50} was calculated to be 3.3 μM.

3.2. *Ion substitution studies provide no evidence for induction of large pores by BzATP*

BzATP is the most potent agonist for activating both native

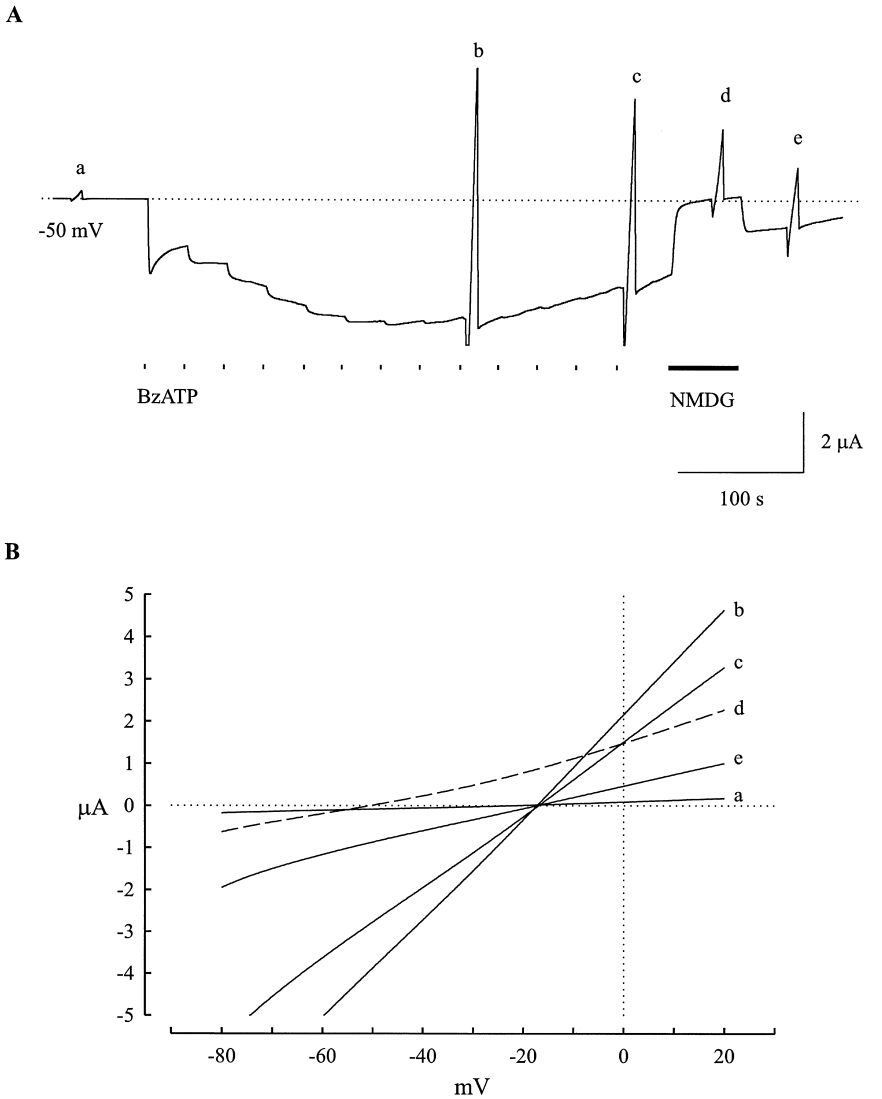

Fig. 4. $NMDG^+$ does not readily permeate those oocytes that have longer lasting rP2X7 responses. A: Thirteen successive 2-s pulses of BzATP (shown as ticks beneath the trace) induced a current that did not desensitize until after many hundreds of seconds. Between applications of BzATP the oocyte was continuously superfused with normal bathing solution. $NMDG^+$-containing superfusate replaced the normal Na^+-containing superfusate as indicated. Inward current is greatly diminished in the presence of $NMDG^+$. Voltage ramps of the same type as those shown in Fig. 3 were applied at 'a' through 'e'. B: Current–voltage plots obtained from ramps 'a' through 'e'. In the presence of $NMDG^+$ the reversal level shifts more than 30 mV in the negative direction, as expected for a greatly decreased permeability to $NMDG^+$ in comparison to inorganic cations.

P2Z receptors [2] and cloned rP2X7 receptors in a number of cell types [3]. BzATP was applied to the *Xenopus* oocytes injected with rP2X7 cRNA, and the resulting currents in solutions containing Na^+ were compared to those in solutions containing the larger organic cations, $NMDG^+$ (MW 195) or $choline^+$ (MW 104). Voltage ramps were applied to obtain measures of conductance and reversal potential. Following the exchange of Na^+ for $NMDG^+$, the BzATP-activated inward current decreased dramatically (Fig. 3A,B) and reversal potentials shifted to a more negative value (Fig. 3C). On average the reversal levels shifted from -12 ± 1.9 mV to -60 ± 3.1 mV ($n = 5$). When $choline^+$ was substituted for Na^+ the reversals shifted from -6.3 ± 4.4 to -27 ± 2.5 mV ($n = 3$). Thus, $NMDG^+$ and $choline^+$ were far less effective current carriers than Na^+, a result inconsistent with the presence of a large pore. (A P_{NMDG}/P_{Na} ratio of 0.15 ± 0.01 ($n = 5$) could be calculated for the rP2X7 receptor from the shift in reversal potential and a $P_{choline}/P_{Na}$ ratio of 0.44 ± 0.04 ($n = 3$) could be calculated in the same way. Such calculations, however, are based on the assumption that current carried by other external ions is negligible.)

Repeated applications of BzATP most often produced the type of responses shown in Fig. 3A,B where the responses grew smaller with repeated applications. In a few oocytes ($n = 4$) repeated applications of BzATP resulted in a current which slowly grew to a maximum and then desensitized very gradually (Fig. 4A.) In such cases superfusion with $NMDG^+$-containing solution in place of Na^+-containing solutions produced a large negative shift in the reversal potential (Fig. 4B). This is inconsistent with the presence of large pores readily permeable to $NMDG^+$.

In several experiments ($n = 9$) the oocytes were injected with

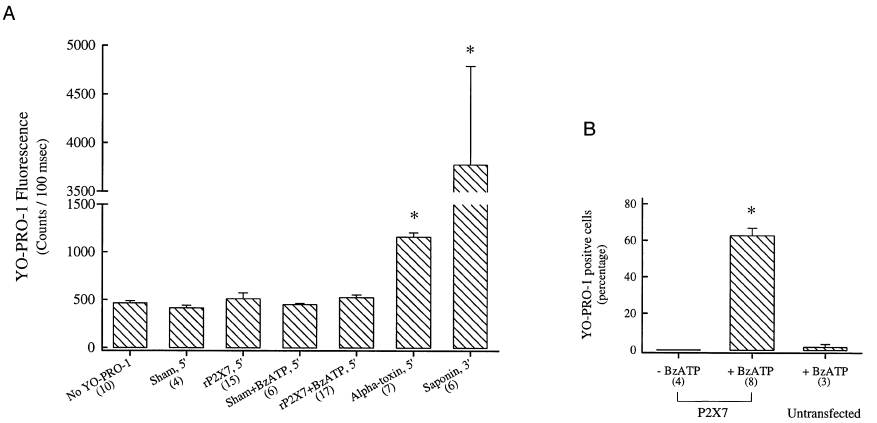

Fig. 5. Failure of oocytes injected with rP2X7 cRNA to accumulate YO-PRO-1^{2+} in response to BzATP application. A: Entry of YO-PRO-1^{2+} into oocytes was monitored by measuring average fluorescence intensity of a region of the oocyte. 'No YO-PRO-1' represents pooled average of sham-injected oocytes and oocytes injected with rP2X7 cRNA in the absence of YO-PRO-1^{2+}. 'Sham, 5'' represents fluorescence of sham-injected oocytes after 5 min in YO-PRO-1^{2+} solution without BzATP. 'Sham+BzATP 5'' represents another group of sham-injected oocytes after 5 min in a solution containing both YO-PRO-1^{2+} and BzATP. 'rP2X7, 5'' and 'rP2X7+BzATP, 5'' give results for oocytes injected with rP2X7 cRNA. BzATP treatment did not increase the measured YO-PRO-1^{2+} entry in the oocytes expressing the rP2X7 receptors. In contrast treatment with *Staphylococcal* alpha-toxin (250 U/ml for 5 min) or saponin (100 μg/ml for 3 min) produced a significant increase in fluorescence intensity when YO-PRO-1^{2+} was present. Graph shows average and SEM for each treatment group. The number in parenthesis indicates the number of oocytes that were used for each observation. B: In HEK293 cells BzATP treatment induces YO-PRO-1^{2+} entry. Summary of results from HEK293 cells stably expressing rP2X7 receptors ('P2X7') compared to untransfected controls ('untransfected'). '-BzATP' indicates those cells that were exposed to YO-PRO-1^{2+} (10 μM) alone for 3 min and '+BzATP' indicates those cells exposed to YO-PRO-1^{2+} with BzATP (100 μM) for 3 min. In each case the percentage of cells showing YO-PRO-1^{2+} fluorescence per microscope field (typically 100 cells or more) was determined, and the results from several different coverslips (number of coverslips shown in parentheses) were averaged and plotted with SEM. Data were compared with multiple *t*-tests using Bonferroni adjustment (GBSTAT vers. 6.0, Dynamic Microsystems Inc.) of α-values for multiple planned comparisons. *Group is significantly different from other groups ($P < 0.05$).

EGTA. We could find no difference in the BzATP-induced currents in oocytes injected with BAPTA compared to those injected with EGTA. A number of experiments were also performed in extracellular solutions containing either zero Ca^{2+} (5 mM EGTA and no added Ca^{2+}; $n = 3$) or 300 μM Ca^{2+} ($n = 3$). Under these conditions BzATP-induced currents were the same as those recorded with 100 μM Ca^{2+} in the extracellular solution.

3.3. *Fluorescent dye measurements provide no evidence for induction of pores by BzATP*

Previous studies have used the entry of the fluorescent monomeric cyanine dye YO-PRO-1 di-iodide (MW of the cyanine cation: 376) as a marker for 'pore' formation induced by activation of native P2Z receptors or the cloned rP2X7 receptor [3]. Upon entry into a cell YO-PRO-1^{2+} binds tightly to nucleic acids which increases its emission intensity and thus allows it to act as an indicator of *cumulative* entry. To test for YO-PRO-1^{2+} permeation, *Xenopus* oocytes were bathed either in a solution containing YO-PRO-1^{2+} (10 μM) alone or a solution containing both YO-PRO-1^{2+} (10 μM) and BzATP (100 μM; free $BzATP^{4-}$, 35 μM). In each case the solution was nominally Mg^{2+}-free and contained 100 μM Ca^{2+}. The fluorescence intensity of sham-injected oocytes and oocytes injected with rP2X7 cRNA was compared after 5 min using a digital imaging microscope. As shown in Fig. 5A, there was no indication that BzATP could increase the entry of YO-PRO-1^{2+} into rP2X7 cRNA injected oocytes. Even after 20 min (data not shown) there was no indication of BzATP-induced YO-PRO-1^{2+} entry in the oocytes expressing the rP2X7 receptors. For the experiments performed on oocytes, positive controls employed the membrane-spanning, pore-producing *Staphylococcal* alpha-toxin, which creates pores that permit entry of molecules up to 3 kDa [11], or the membrane permeabilizing agent saponin [10]. In the presence of these agents (Fig. 5A), YO-PRO-1^{2+} entry into the oocytes could be monitored readily.

When similar experiments were performed with HEK293 cells stably transfected with rP2X7 (kindly supplied by George Dubyak), BzATP-activated YO-PRO-1^{2+} entry was clearly present (Fig. 5B), confirming the earlier observation that in HEK293 cells BzATP induces a non-selective pathway permeable to YO-PRO-1^{2+} [3].

In summary, two independent lines of experimentation, electrophysiological analyses of ion currents and fluorescent measurements of YO-PRO-1^{2+} dye entry, failed to disclose evidence of pore formation in *Xenopus* oocytes expressing cloned rP2X7 receptors.

4. Discussion

With the cloning and characterization of the rP2X7 receptor by Surprenant et al. [3], two long-standing lines of investigation converged. The first stems from the hypothesis originally put forward by Burnstock [12] that ATP acts as a neurotransmitter which activates ligand-gated cation channels that came to be known as P2X receptors. The second line of study arises from the observation of Cockroft and Gomperts [13,14] that mast cells could be reversibly permeabilized to molecules much larger than organic cations by ATP^{4-}, the receptors for which are designated as P2Z receptors. Surprenant et al. [3] have now shown that a variety of cells transfected with rP2X7 cDNA can give rise to P2Z-like or 'pore-forming' responses when treated with ATP or BzATP, an observation that we have confirmed in this report. The results in the present study suggest that factors in addition to the rP2X7

receptor are involved in the pore-forming response since *Xenopus* oocytes injected with rP2X7 cRNA express ATP-gated cation channels but not larger pores of the P2Z type.

What could the additional factor or factors be? Our original hypothesis was that the P2X4 subtype, when co-expressed with P2X7, might form a complex which would inhibit the formation of larger pores. This idea arose from our work on a P2X receptor in smooth muscle cells from toad stomach which gave rise to cationic currents and which had a pharmacological profile resembling P2Z receptors in many respects [10]. However, this receptor did not give rise to larger pores. Although this receptor from toad has not been cloned, a similar response was observed some time ago by Honore et al. [15] in pregnant rat myometrial cells. Poly(A)$^+$ RNA which we prepared from such myometrial cells was analyzed using PCR by Buell and his colleagues, and both P2X4 and P2X7 subtypes were found (unpublished observations). Moreover, Juranka et al. [16] recently screened a *Xenopus* oocyte cDNA library and found five cDNAs, designated xP2X4a-e, with a 67% predicted amino acid identity to rat P2X4 receptors. It is possible that one of these xP2X4 subunits serves to inhibit the formation of P2Z-like receptors from the P2X7 subtype, perhaps by forming a heterooligomer.

However, there is reason to believe that a factor other than the P2X4 subtype, or in addition to it, is at work. Nuttle and Dubyak [4] have studied P2Z responses in the murine macrophage cell line BAC1.2F5 which has P2X7 mRNA (Dubyak, personal communication). When *Xenopus* oocytes are injected with poly(A)$^+$ RNA derived from the murine BAC1.2F5 cells, P2Z-like responses are seen. These results combined with the results in the present study suggest that there is a factor coded for by the BAC1.2F5 murine macrophage mRNA which is necessary for the P2Z response, i.e. a facilitatory as opposed to an inhibitory agent.

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