5-Nitro-2-(3-phenylpropylamino)benzoic Acid (NPPB) Stimulates Cellular ATP Release through Exocytosis of ATP-enriched Vesicles*

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Cells release ATP in response to physiologic stimuli. Extracellular ATP regulates a broad range of important cellular functions by activation of the purinergic receptors in the plasma membrane. The purpose of these studies was to assess the role of vesicular exocytosis in cellular ATP release. FM1-43 fluorescence was used to measure exocytosis and bioluminescence to measure ATP release in HTC rat hepatoma and Mz-Cha-1 human cholangiocarcinoma cells. Exposure to a Cl⁻ channel inhibitor 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (50–300 μM) stimulated a 5–100-fold increase in extracellular ATP levels within minutes of the exposure. This rapid response was not a result of changes in cell viability or Cl⁻ channel activity. NPPB also potently stimulated ATP release in HEK293 cells and HEK293 cells expressing a rat P2X7 receptor indicating that P2X7 receptors are not involved in stimulation of ATP release by NPPB. In all cells studied, NPPB rapidly stimulated vesicular exocytosis that persisted many minutes after the exposure. The kinetics of NPPB-evoked exocytosis and ATP release were similar. Furthermore, the magnitudes of NPPB-evoked exocytosis and ATP release were correlated (correlation coefficient 0.77), indicating that NPPB may stimulate exocytosis of a pool of ATP-enriched vesicles. These findings provide further support for the concept that vesicular exocytosis plays an important role in cellular ATP release and suggest that NPPB can be used as a biochemical tool to specifically stimulate ATP release through exocytic mechanisms.

Extracellular ATP acts as a potent signaling molecule in many different tissues including the immune system, neurons, endothelial cells, and secretory epithelia by activation of purinergic receptors in the plasma membrane (1). Cells release ATP in response to physiologic stimuli such as shear stress, stretch, osmotic swelling, and hypoxia (2–5). One mechanism for ATP release involves movement of ATP through transporters or channel proteins in the plasma membrane. There is evidence for ATP release through ATP-binding cassette (ABC) transporters, connexin and pannexin hemichannels, P2X7-pannexin1 receptor-channel complex, and multiple Cl⁻ channels (6–11). In addition, there is evidence for exocytic vesicular release of ATP (4, 12). Under basal conditions, the concentration of ATP in extracellular medium is in the low nanomolar range. Vesicles store ATP in the millimolar range, and exocytosis of these ATP-enriched vesicles increases local ATP concentrations. It has been difficult to study the contribution of exocytosis in ATP release because many cells are capable of releasing ATP through more than one mechanism. For example, ATP release from astrocytes is mediated by both vesicular exocytosis and transport proteins (8, 13–15). Thus, the role of vesicular exocytosis in ATP release is still poorly understood.

5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB)² is widely used as an inhibitor of many different Cl⁻ channels and has been reported to inhibit ATP release mediated by Cl⁻ channels (16, 17). In some cells, NPPB also inhibits ATP release mediated by mechanosensitive and pannexin1 channels, and other channels that have not been defined (2, 10, 18, 19). Thus, NPPB is an inhibitor of channel-mediated ATP release.

The purpose of these studies was to assess the role of vesicular exocytosis in cellular ATP release. Using FM1-43 fluorescence to measure exocytosis and bioluminescence assay to measure ATP release in real time, we found that exposure to NPPB under basal conditions potently stimulates ATP release. These previously unknown effects of NPPB appear to be mediated through stimulation of exocytosis of a pool of ATP-enriched vesicles.

**EXPERIMENTAL PROCEDURES**

**Cell Models**—Studies of ATP release were performed in HTC and Mz-Cha-1 cells. Both cell lines have been utilized as models for cellular ATP release, degradation, and purinergic signaling in secretory epithelia (7, 20). HTC cells are derived from rat hepatoma, and Mz-Cha-1 cells are derived from human adenocarcinoma of the gall bladder. The procedures for culturing these cells have been previously described (21, 22). Cells were used within 48 h after plating.

**Measurement of ATP Release**—Cellular release of ATP was measured using the luciferin-luciferase assay as previously described (23, 24). All cells were grown to confluence in 35-mm Petri dishes. Prior to study, cells were gently washed twice with 1 ml of OptiMEM (Invitrogen) and then 800 μl of OptiMEM containing 2 mg/ml firefly luciferin-luciferase (Sigma cat. num. 33894 JOURNAL OF BIOLOGICAL CHEMISTRY

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² The abbreviations used are: NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; HEK293, human embryonic kidney; EGFP, enhanced green fluorescent protein; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; FM1-43, N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; COX, cyclooxygenase; CBX, carbenoxolone; MFQ, melphalan.

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Solutions for Imaging—All imaging experiments were performed after washing culture medium with a standard physiologic solution that contained 142 mM NaCl, 4 mM KCl, 1 mM KH₂PO₄, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM Hepes, 10 mM d-glucose. The pH was 7.25, and osmolarity was 295–305 mosmol/kg. All compounds were purchased from Sigma-Aldrich (St. Louis, MO).

Imaging and Analysis—For imaging experiments, cells were plated on coverslips in the recording chambers and incubated overnight in the culture medium as described above. Cells were washed with a standard physiologic solution, and visualized through a 40× oil immersion objective (NA = 1.35) and an Olympus IX71 microscope. The images were acquired with a SensiCam QE camera controlled by a SlideBook 3.0 software (Intelligent Imaging Innovations, Denver, CO). The cellular fluorescence was measured by drawing a region of interest over the entire cell and subtracting background fluorescence. The background fluorescence was measured from the regions with no cells.

Measurement of Cell Viability—Cell viability was determined by measuring calcein fluorescence. Calcein AM permeates cell membranes, and once inside the cell, this nonfluorescent substrate is converted by cellular esterases into fluorescent negatively charged calcein that is retained by cells with intact plasma membranes. In contrast, calcein rapidly leaks from dead or damaged cells with compromised membranes. For these experiments, cells were incubated with 1 μM calcein AM (Invitrogen, Carlsbad, CA) dissolved in DMSO for 10–30 min at 37 °C. Prior to measurements, the dye was removed from the extracellular solution. The fluorescence was excited through an excitation filter (peak 480 nm) and collected with an emission filter (peak 535 nm). Calcein fluorescence (F) was measured every 20 s using exposures of 15 ms and expressed as F/F₀ where F₀ is the initial fluorescence at t = 0 min.

In the absence of calcein, no signal was detected in HEK293 or HEK293 cells overexpressing a rat P2X7 receptor tagged with an enhanced green fluorescent protein (EGFP) (HEK293-P2X7). The fluorescence of calcein was about 20-fold larger than the fluorescence of EGFP. This feature permitted the measurement of cell viability in HEK293-P2X7 cells.

Immunolocalization of P2X7 Receptors—Cellular localization of the P2X7 receptors in HTC and Mz-Cha-1 cells was
performed using immunofluorescence. All incubations were performed at room temperature. Briefly, cells were plated on the coverslips, fixed, and permeabilized with PBS containing 10% paraformaldehyde and 0.01% Tween 20 for 30 min. After washing, cells were incubated with primary P2X7 ectoAb (Alomone Labs, Jerusalem, Israel) at 1:100 in PBS/0.1% bovine serum albumin (BSA) for 1 h. Cells were then washed and incubated with secondary fluorescent antibody (Alexa-Fluor 546 donkey anti-rabbit IgG, Invitrogen) at 1:100 in PBS/0.1% BSA for 1 h. To visualize cell nucleus, cells were incubated with 0.5 μg/ml DAPI (Invitrogen) for 5 min. Cells were imaged using a confocal microscope Leica TCS SP5 and an oil immersion objective (63×, NA = 1.4). The P2X7 ectoAb staining was detected with an excitation/emission filter (561/610 nm), and an excitation/emission filter (405/452 nm) was used for detection of DAPI nuclear staining.

Detection of P2X7 receptors using the P2X7 ectoAb is cell specific (25). To determine whether this antibody also binds to other proteins in HTC and Mz-Cha-1 cells, control experiments were performed by incubating cells with a purified nonspecific isotype IgG antibody (Jackson Immunoresearch Labs, West Grove, PA) and secondary fluorescent antibody.

**Cell Transfection**—HEK293 cells were used for overexpression of P2X7 receptors, and were routinely maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (HyClone), 1% penicillin-streptomycin solution, and 1% L-glutamine (Invitrogen). HEK293 cells were transfected with a plasmid containing EGFP expression vector (pIRES2-EGFP) and a rat P2X7 receptor (kindly provided by S. Stojilkovic). Transfection was conducted 24 h after plating the cells using 2 μg of DNA and 5 μl of Lipofectamine 2000 reagent (Invitrogen) in 10 ml of serum-free Opti-MEM. After 4.5–6 h of incubation, the transfection mixture was replaced with normal culture medium, and cells were cultured for an additional 24–48 h. Transfected cells were mechanically dispersed and re-cultured with Geneticin G418 sulfate (Invitrogen) for 24–48 h. After selection, transfected cells were cultured in 35-mm Petri dishes for studies of ATP release and recording chambers for imaging experiments.

**Measurement of Protein Content**—To account for the variability in the number of cells among individual dishes, the total cellular protein content was determined by a bicinchoninic acid (BCA) protein assay as described previously (26). The BCA assay (Sigma-Aldrich) was performed according to the manufacturer’s instructions by measuring absorbances of the samples at 562 nm after incubation at 37 °C for 30 min. The concentration of protein in the sample (in μg/ml) was determined using the standard curve of absorbance versus bovine serum albumin protein sample concentration.

**Measurement of Exocytosis**—The magnitude of exocytosis was measured using a fluorescent dye N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide (FM1-43) as previously described (27). FM1-43 binds to membranes but does not permeate through lipid bilayers. The dye is not fluorescent in solution, but it becomes fluorescent upon

![FIGURE 2. The effects of NPPB on viability of HTC cells.](image-url) HTC cells were preincubated with 1 μM calcein AM for 30 min. After washing the dye from extracellular solution, calcein fluorescence was measured every 20 s. At the arrow, cells were exposed to 300 μM NPPB (n = 7 cells) or water (n = 4 cells). Note that NPPB does not change calcein fluorescence.

![FIGURE 3. Localization of P2X7 receptors.](image-url) HTC and Mz-Cha-1 cells were fixed and permeabilized, and then incubated with primary P2X7 ectoAb (left panels) or control nonspecific isotype IgG antibody (right panels). To visualize the cellular distribution of P2X7 receptors, cells were stained with secondary fluorescent antibody (red) and the cell nucleus was labeled with DAPI (blue). Bright field and confocal fluorescent images were superimposed. Almost no red staining was obtained with control IgG indicating that binding of the P2X7 ectoAb to other proteins is minimal. Note that P2X7 receptors are present in both cells, and the P2X7 staining is detected in the cytosol and the plasma membrane.
binding to the biological membranes (28). In the presence of FM1-43 in the extracellular solution, exocytic insertion of vesicles into the plasma membrane results in staining of the vesicle membrane and an increase in FM1-43 fluorescence. Thus, the overall change in FM1-43 fluorescence provides in real time a measure of the sum of all exocytic events (29, 30). For these experiments, cells were stained with 4 μM FM1-43 (Invitrogen). The fluorescence was excited with an excitation filter (peak 480 nm) and collected with an emission filter (peak 535 nm). FM1-43 fluorescence was measured every 30 s using exposures of 200 ms. The initial staining of the plasma membrane was used to determine a baseline fluorescence (100%). The magnitude of exocytosis (ΔF) was determined as a change from baseline fluorescence (in percent) 10 min after exposure to NPPB.

FM1-43 has a broad excitation and emission spectra, and the changes in FM1-43 fluorescence can be also measured using an excitation filter (peak 535 nm) and an emission filter (peak 610 nm). This approach was used to measure exocytosis in HEK293-P2X7 cells. Initially, the P2X7-positive cells were identified using a 480/535 nm excitation/emission filter. Subsequently, FM1-43 fluorescence was measured from these cells using a 535/610 nm excitation/emission filter.

Statistics—Data were expressed as mean ± S.E. Results were compared using a two-tailed Student’s t test on paired and unpaired data, and p values < 0.05 were considered to be statistically significant.

RESULTS

NPPB Stimulates ATP Release

To assess the effects of NPPB on ATP release, the amount of ATP in the medium was measured using luminescence in HTC and Mz-Cha-1 cells. Representative recordings in Fig. 1A illustrate that exposure to NPPB rapidly increased luminescence in both cells by about 5–20-fold, and this stimulatory effect per-

FIGURE 4. NPPB stimulates ATP release in HEK293 cells. A, HEK293 cells were incubated with a plasmid containing an EGFP vector and a rat P2X7 receptor (bottom left panel). These cells exhibit evenly distributed fluorescence as indicative of the P2X7 receptor expression. No fluorescence was observed in HEK293 cells (top left panel). Middle panels show bright field images of cells in the left panels under control conditions. Right panels show the same cells 4 min after exposure to 100 μM BzATP. Note that BzATP induced the formation of membrane blebs in HEK293-P2X7 cells. Interestingly, a population of cells with the highest expression level exhibited a decrease in the number of membrane blebs. B, luminescence was measured after addition of 100 μM NPPB (at the arrow) in a HEK293 cell (open circles) and a HEK293-P2X7 cell (closed circles). C, peak of relative luminescence in response to different NPPB concentrations was measured in HEK293 cells (open circles) and HEK293-P2X7 cells (closed circles). To correct for the decreased sensitivity of the assay in the presence of NPPB, relative luminescence was divided by a factor obtained from the data in Fig. 1B. DMSO (0.1%) did not significantly increase luminescence in HEK293 cells (open triangles) or HEK293-P2X7 cells (closed triangles). An inset shows the dose response on larger scale. The number of dishes analyzed was from 2 to 13.
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FIGURE 5. The effects of NPPB on viability of HEK293 cells. Calcein fluorescence was measured in HEK293 (open circles) and HEK293-P2X7 cells (closed circles). A, addition of 100 μM NPPB (at the arrow) did not significantly change calcein fluorescence in HEK293 cells (n = 5) or HEK293-P2X7 cells (n = 7). B, exposure to 100 μM BzATP (at the arrow) significantly decreased calcein fluorescence in HEK293-P2X7 cells (n = 5), but had no effect in HEK293 cells (n = 10). Addition of probenecid (1 mM) 5 min before BzATP further stimulated a loss of calcein fluorescence (n = 5 cells). C, loss of calcein fluorescence was measured in HEK-P2X7 cells 15 min after exposure to BzATP. The inhibitors of pannexin1 channels were added 5 min prior to BzATP. BzATP-evoked loss of calcein was increased with probenecid (1 mM, p < 0.01). No significant effect was found with MFQ (10 μM), CBX (100 μM), or NPPB (100 μM). The number of cells analyzed was from 4 to 6.

NPPB has also been reported to inhibit cyclooxygenase (COX) (32). To assess the potential role of COX, ATP release was measured after addition of indomethacin. Indomethacin ATP (Fig. 1B) (2). Thus, the stimulatory effects of NPPB in Fig. 1A are not related to the modulation of luciferin-luciferase assay, but rather they result from stimulation of cellular ATP release. Fig. 1C shows that NPPB-stimulated ATP release in HTC and Mz-Cha-1 cells in a dose-dependent manner. Because of a substantial loss of sensitivity at higher concentrations, it was not possible to determine the EC50. Furthermore, because NPPB was dissolved in DMSO, the effect of DMSO on ATP release was evaluated. DMSO had no effect on the luciferin-luciferase assay (not shown) or ATP release in both cells (triangles, Fig. 1C). These results indicate that NPPB stimulates ATP release from HTC and Mz-Cha-1 cells in a dose-dependent manner.

To assess whether ATP release evoked by NPPB was caused by disruption of the plasma membrane and a leakage of ATP into extracellular solution, cell viability was measured using calcein fluorescence. Fig. 2 shows that NPPB had no effect on calcein fluorescence in HTC cells. When the plasma membrane was ruptured by exposing cells to water, the fluorescence decreased to zero as a result of calcein leakage out of the cell (Fig. 2). Similar results were obtained in Mz-Cha-1 cells (not shown). Thus, it is unlikely that NPPB-evoked ATP release is mediated by changes in cell viability.

Role of Cl−/H11002 Channels and COX—
NPPB is a potent inhibitor of Cl−/H11002 channels in HTC and Mz-Cha-1 cells (21, 31). To assess whether inhibition of Cl− channels is involved in NPPB-evoked ATP release, cells were exposed to another Cl− channel inhibitor 4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid (DIDS). Similar to NPPB, exposures to DIDS decreased the sensitivity of luciferin-luciferase to ATP (not shown). However, DIDS (100 μM) decreased relative luminescence to 0.95 ± 0.10 (n = 4 dishes) and 0.65 ± 0.10 (n = 2 dishes) in HTC and Mz-Cha-1 cells, respectively.

NPPB has been also reported to inhibit cyclooxygenase (COX) (32). To assess the potential role of COX, ATP release was measured after addition of indomethacin. Indomethacin...
presence of P2X7 receptors using immunofluorescence. Representative images in Fig. 3 illustrate that P2X7 receptors are present in both HTC and Mz-Cha-1 cells. However, because P2X7 receptors have not been functionally characterized in these cells and many P2X7 antagonists have other non-P2X7 receptor-mediated effects, we used a different strategy to assess the role of P2X7 receptors (33, 34).

ATP release was measured in HEK293 cells that overexpress a rat P2X7 receptor. The P2X7 receptors were tagged with EGFP, and following transfection, EGFP fluorescence was detected in ~80% of cells (Fig. 4A, left panels). Exposure to a P2X7 agonist BzATP induced the formation of membrane blebs in HEK293-P2X7 cells but not in HEK293 cells (Fig. 4A, middle and right panels), indicating that the plasma membrane P2X7 receptors are functional, consistent with previous reports using the same plasmid (35, 36). Basal ATP levels in HEK293-P2X7 cells (547,546 ± 12,724 counts, n = 23 dishes) were significantly larger than in HEK293 cells (333,730 ± 7,584 counts, n = 37 dishes, p < 0.001). To assess whether this increase was caused by differences in the number of cells, the total protein content in the dish was measured. The total protein content in HEK293-P2X7 cells (0.68 ± 0.04 g/ml, n = 19 dishes) was significantly lower than in HEK293 cells (0.98 ± 0.12 g/ml, n = 17 dishes, p < 0.02). Thus, overexpression of P2X7 receptors induces a cellular phenotype with the increased capacity for ATP release under basal conditions.

FIGURE 6. NPPB stimulates exocytosis in HTC and Mz-Cha-1 cells. A, fluorescent images of cells obtained after staining the plasma membrane with FM1-43 are shown in left panels. Exposure to 200 μM NPPB for 10 min stimulated exocytosis in both cells (middle panels). After washing the dye and NPPB, the remaining FM1-43 fluorescence was localized in the intracellular compartments as indicative of endocytosis that occurred in the presence of the dye (right panels). B, FM1-43 fluorescence was measured in HTC cells after addition of 100 μM NPPB (n = 5 cells, closed circles) or 0.1% DMSO (n = 4 cells, open circles). The drugs were applied at the arrow. C, recordings of FM1-43 fluorescence in Mz-Cha-1 cells after exposure to 100 μM NPPB (n = 4 cells, closed circles) or 0.1% DMSO (n = 5, open circles). D, magnitude of exocytosis (ΔF) was measured 10 min after exposure to different concentrations of NPPB in HTC cells (open circles) and Mz-Cha-1 cells (closed circles). The ΔF was also measured after exposure to 0.1% DMSO in HTC cells (open triangles) and Mz-Cha-1 cells (closed triangles). The number of cells analyzed was from 3 to 17.

(100 μM) did not affect the sensitivity of luciferin-luciferase to ATP (not shown), but decreased relative luminescence to 0.90 ± 0.04 (n = 4 dishes) and 0.70 ± 0.04 (n = 2 dishes) in HTC and Mz-Cha-1 cells, respectively. These data suggest that stimulation of ATP release by NPPB can be dissociated from inhibition of Cl− channels or COX activity.

Role of P2X7 Receptors—To assess whether P2X7 receptors are involved in NPPB-evoked ATP release, we looked for the dependence of NPPB-evoked ATP release in both cells was similar (Fig. 4C). These findings suggest that P2X7 receptors are not required for stimulation of ATP release by NPPB.

To assess the role of pannexin1, ATP release was measured after inhibition of pannexin1 channels with carbenoxolone (CBX). The peak of relative luminescence evoked by 100 μM NPPB was not different in the absence or presence of 100 μM CBX in HEK293 cells (p > 0.58, n = 4) and HEK293-P2X7 cells

FIGURE 6. NPPB stimulates exocytosis in HTC and Mz-Cha-1 cells. A, fluorescent images of cells obtained after staining the plasma membrane with FM1-43 are shown in left panels. Exposure to 200 μM NPPB for 10 min stimulated exocytosis in both cells (middle panels). After washing the dye and NPPB, the remaining FM1-43 fluorescence was localized in the intracellular compartments as indicative of endocytosis that occurred in the presence of the dye (right panels). B, FM1-43 fluorescence was measured in HTC cells after addition of 100 μM NPPB (n = 5 cells, closed circles) or 0.1% DMSO (n = 4 cells, open circles). The drugs were applied at the arrow. C, recordings of FM1-43 fluorescence in Mz-Cha-1 cells after exposure to 100 μM NPPB (n = 4 cells, closed circles) or 0.1% DMSO (n = 5, open circles). D, magnitude of exocytosis (ΔF) was measured 10 min after exposure to different concentrations of NPPB in HTC cells (open circles) and Mz-Cha-1 cells (closed circles). The ΔF was also measured after exposure to 0.1% DMSO in HTC cells (open triangles) and Mz-Cha-1 cells (closed triangles). The number of cells analyzed was from 3 to 17.
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(p > 0.48, n = 4). These results suggest that pannexin1 channels and/or a P2X7-pannexin1 receptor channel complex are not involved in NPPB-evoked ATP release.

To assess whether NPPB has an effect on viability of HEK293 cells, calcine fluorescence was measured after exposure to NPPB. Fig. 5A shows that NPPB had no effect on calcine fluorescence in HEK293 or HEK293-P2X7 cells, indicating that NPPB does not increase extracellular ATP concentration by disrupting the plasma membrane. Further experiments revealed an interesting property of calcine. BzATP had no effect on calcine fluorescence in HEK293 cells, but slowly decreased calcine fluorescence in HEK293-P2X7 cells (Fig. 5B). Because stimulation of P2X7 receptors is associated with activation of a dye-permeable pathway that allows passage of large molecules across the plasma membrane, it is likely that calcine (molecular mass, ~620 Da) permeates through a P2X7 receptor-activated pore (37). Pharmacological studies have indicated that slow permeation of calcine through a P2X7 receptor-activated pore is independent of activation of pannexin1 channels. Probencid increased BzATP-evoked loss of calcine (Fig. 5, B and C). Other inhibitors of pannexin1 channels, mefloquine (MFQ), CBX, and NPPB did not significantly change calcine loss in response to BzATP (Fig. 5C). Recent studies have described a slow pannexin1-independent dye-permeable pathway in HEK293-P2X7 cells (38). Thus, calcine may be potentially useful to measure activation of this slow pathway.

NPPB-dependent Exocytosis—To assess whether NPPB stimulates vesicular exocytosis, FM1-43 fluorescence was measured after exposure to NPPB. Representative images in Fig. 6A illustrate that NPPB markedly increased FM1-43 fluorescence in both HTC and Mz-Cha-1 cells. If NPPB stimulates the fluorescence of membrane-bound FM1-43, then it would increase FM1-43 fluorescence transiently, and no further increase would be observed in the absence of exocytosis. Quantitative analysis in Fig. 6, B and C shows that NPPB stimulated a slow gradual increase in FM1-43 fluorescence that persisted many minutes after the exposure. Thus, it is unlikely that NPPB stimulates the fluorescence of membrane-bound FM1-43. A gradual increase in FM1-43 fluorescence suggests that NPPB stimulates continuous vesicular exocytosis. To further examine the effect of NPPB, the dye and NPPB were washed from the extracellular solution. After the dye was removed from the plasma membrane, substantial FM1-43 fluorescence was detected in the cytosol (right panels in Fig. 6A). In contrast to NPPB, FM1-43 is not membrane permeable, and the residual fluorescence comes from the dye trapped through endocytosis in the intracellular compartments. Moreover, an increase in FM1-43 fluorescence that occurred in the presence of NPPB was not different from the fluorescence that remained after washing in HTC cells (p > 0.31, n = 5 cells) and Mz-Cha-1 cells, (p > 0.12, n = 5 cells). These data indicate that NPPB stimulates vesicular exocytosis, which is followed by compensatory endocytosis, and this activity persists many minutes after exposure to NPPB.

The data in Fig. 6, B and C show that DMSO also increased FM1-43 fluorescence. However, these changes were about 10–20% in 10 min, which correspond to the rates of exocytosis of 1–2% per min that have been previously found in HTC and Mz-Cha-1 cells and result from constitutive exocytosis under basal conditions (27, 39). Fig. 6D shows that the magnitude of NPPB-evoked exocytosis was concentration dependent in both HTC and Mz-Cha-1 cells.

To assess whether NPPB stimulates exocytosis through mechanisms involving P2X7 receptors, FM1-43 fluorescence was measured in HEK293-P2X7 cells. Exposure to NPPB potently increased FM1-43 fluorescence in both HEK293 and
HEK293-P2X7 cells, and the rates of FM1-43 fluorescence remained elevated many minutes after exposure (Fig. 7, A and B). Fig. 7C shows that dose-response curves of NPPB-evoked exocytosis in HEK293 and HEK293-P2X7 cells were similar. Thus, stimulation of exocytosis by NPPB does not require signaling through P2X7 receptors.

Collectively, these data indicate that NPPB stimulates both vesicular exocytosis and ATP release with similar kinetics and dose dependence in all cells studied (compare Figs. 1 and 6, also Figs. 4 and 7). When the magnitude of exocytosis was plotted versus relative luminescence obtained from all cells with NPPB (50–200 μM), the larger exocytic responses were associated with larger ATP release (correlation coefficient: 0.77, Fig. 8). The magnitude of exocytosis was not correlated with ATP release for 300 μM NPPB (inset in Fig. 8). These results suggest that NPPB may stimulate cellular ATP release in part by exocytosis of a pool of ATP-enriched vesicles.

**DISCUSSION**

NPPB is widely used as a potent inhibitor of many different Cl⁻ channels. Consequently, NPPB inhibits Cl⁻ channel-mediated ATP release. The major finding of these studies is that NPPB is also able to stimulate cellular ATP release through mechanisms that are independent of the activity of Cl⁻ channels. These novel effects of NPPB appear to be mediated in part by exocytosis of a pool of ATP-enriched vesicles. Thus, NPPB may be used as a biochemical tool to specifically stimulate ATP release through vesicular exocytosis.

The effects of NPPB on ATP release were studied in HTC and Mz-Cha-1 cells. These cells have been used as models for other mechanisms of ATP release. However, the dose response of NPPB-evoked ATP release does not support this hypothesis. Whereas NPPB concentrations required to stimulate ATP release were >50 μM, NPPB potently inhibits Cl⁻ channels at low micromolar concentrations (44). At these concentrations, NPPB had almost no effect on ATP release in all cells studied here (see Figs. 1C and 4C). These results provide strong evidence that Cl⁻ channels are not involved in stimulation of ATP release by NPPB.

Exposure to NPPB potently stimulated vesicular exocytosis within minutes of exposure, and this exocytic activity persisted many minutes after the exposure in each cell type tested. Notably, the kinetics of NPPB-evoked exocytosis and ATP release were similar, and stimulation of exocytosis also required NPPB concentrations of >50 μM. These findings provide support for the concept that NPPB may act by mobilizing a pool of ATP-enriched vesicles and sustained exocytosis of these vesicles may mediate ATP release. The data in Fig. 8 provide support for this concept by showing that the magnitudes of exocytosis, and ATP release were correlated. However, at the highest concentration of NPPB tested (300 μM), the correlation was lost. The reasons for this discrepancy are not known for certain. One explanation may be that NPPB stimulates ATP release through mechanisms other than exocytosis, and these mechanisms become dominant only at high concentrations. Another explanation may be that a pool of ATP-enriched vesicles is limited. Thus, if exocytic vesicles stained with FM1-43 are endocytosed and refilled with ATP and subsequently undergo the next round of exocytosis, then ATP release from these vesicles would not further increase FM1-43 fluorescence. Indeed, expo-
sures to 300 μM NPPB stimulated massive 50–200-fold increase in ATP release (see inset in Fig. 8). Consistent with this explanation, when the dye was removed from the plasma membrane, NPPB-evoked increases in FM1-43 fluorescence were detected in the cytosol (see Fig. 6A). These results are indicative of ongoing endocytosis and suggest that NPPB may stimulate trafficking of a pool of ATP-enriched vesicles. Assuming that constitutive membrane trafficking of a pool of ATP-enriched vesicles contributes to ATP release under basal conditions; then NPPB may act to rapidly recruit new vesicles into this pool and/or mobilize these vesicles to undergo fusion with the plasma membrane.

Assuming that vesicular exocytosis is important for NPPB-evoked ATP release, several important points merit emphasis. First, NPPB may also stimulate insertion of ATP transport proteins into the plasma membrane and/or activate these proteins already present in the cell membrane. We used HEK293 transfected with a rat P2X7 receptor to assess the potential contribution of P2X7 receptors. These proteins were selected because they contribute to membrane ATP permeability and regulate a broad range of membrane-trafficking responses (14, 45, 46). As expected, BzATP induced the formation of membrane blebs in HEK293-P2X7 cells and not in HEK293 cells. However, a population of HEK293-P2X7 cells with the highest expression level exhibited a decrease in the number of membrane blebs (see Fig. 4A). The reasons for this discrepancy are not known. One possible explanation may be that high expression levels of P2X7 receptors in the cytosol decrease the number of P2X7 receptors in the plasma membrane. Another explanation may be that P2X7 receptor crowding in the plasma membrane stimulates tyrosine phosphorylation of a heat shock protein 90 within the P2X7 receptor complex. This phosphorylation has been shown to inhibit the P2X7 receptor function (47). In any case, previous studies have demonstrated that P2X7 receptors contribute to ATP release in HEK293-P2X7 cells under basal conditions (48). We also found that HEK293-P2X7 cells released more ATP than HEK293 cells under basal conditions. However, the effects of NPPB were observed in both HEK293 and HEK293-P2X7 cells, indicating that P2X7 receptors are not required for stimulation of ATP release or vesicular exocytosis by NPPB.

Second, it has been proposed that permeation of large molecules through P2X7 receptors requires the presence of pannexin1 (9). There is also evidence that pannexin1 per se forms an ion channel permeable to ATP (49, 50). Whereas HEK293 cells express pannexin1, it is not known whether HTC and Mz-Cha-1 cells express pannexin1 (9). In any case, it is unlikely that NPPB-evoked ATP release is mediated through pannexin1 channels, because CBX had no effect on NPPB-evoked ATP release in both HEK293 and HEK293-P2X7 cells.

Finally, the cellular mechanisms responsible for exocytosis of ATP-enriched vesicles by NPPB are not known. Because NPPB is membrane permeable, it is not known whether NPPB acts from the extracellular and/or intracellular site. We have previously demonstrated that insulin stimulates exocytosis of Cl− channel-containing vesicles, and this response leads to an increase in membrane Cl− permeability in HTC cells (27). However, insulin had no effect on ATP release in HTC cells.3 Thus, it is attractive to speculate that multiple vesicle pools exist, and exocytosis of these vesicles may be important for shaping the cellular response to different physiologic stimuli. In summary, these findings provide further support for the concept that vesicular exocytosis plays a key role in the regulation of cellular ATP release and suggest that NPPB may be used as a biochemical tool to specifically stimulate ATP release through exocytosis of a pool of ATP-enriched vesicles.

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