Receptor-independent effects of 2'(3')-O-(4-benzoylbenzoyl) ATP triethylammonium salt on cytosolic pH

Juan Pablo Reyes · Matthew W. Grol ·
Stephen M. Sims · S. Jeffrey Dixon

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Abstract The effect of the relatively potent P2X7 receptor agonist 2'(3')-O-(4-benzoylbenzoyl)adenosine 5'-triphosphate triethylammonium salt (BzATP-TEA) on cytosolic pH (pH_i) was studied using MC3T3-E1 osteoblast-like cells, which endogenously express P2X7 receptors. pH_i was measured fluorimetrically using the pH-sensitive dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein. BzATP-TEA (0.3–1.5 mM) elicited fast-onset alkalinization responses. In contrast, adenosine 5'-triphosphate disodium salt (5 mM) failed to reproduce the BzATP-TEA-induced responses, indicating a P2 receptor-independent mechanism. We speculated that triethylamine, which is present in solutions of BzATP-TEA, permeates the plasma membrane, and is protonated intracellularly, leading to an increase in pH_i . Consistent with this hypothesis, triethylammonium (TEA) chloride mimicked the effects of BzATP-TEA on pH_i . Moreover, measurements using a Cytosensor microphysiometer revealed that TEA chloride transiently suppressed proton efflux from cells, whereas washout of TEA transiently enhanced proton efflux. BzATP-TEA also elicited a sustained increase in proton efflux that was blocked specifically by the P2X7

antagonist A-438079. Taken together, we conclude that BzATP-TEA-induced alkalinization is unrelated to P2X7 activation, but is due to the presence of TEA. This effect may confound assessment of the outcomes of P2X7 activation by BzATP-TEA in other systems. Thus, control experiments using TEA chloride are recommended to distinguish between receptor-mediated and nonspecific effects of this widely used agonist. We performed such a control and confirmed that BzATP-TEA, but not TEA chloride, caused the elevation of cytosolic free Ca^{2+} in MC3T3-E1 cells, ruling out the possibility that receptor-independent effects on pH_i underlie BzATP-TEA-induced Ca^{2+} signaling.

Keywords Cytosolic calcium · Cytosolic pH · Microphysiometer · P2X7 · Proton efflux · Triethylamine

Introduction

Stimulation of P2 nucleotide receptors present in the plasma membrane of mammalian cells with adenosine 5'-triphosphate (ATP) or other agonists elicits a variety of responses [1]. Among the P2 receptor agonists utilized in contemporary research, 2'(3')-O-(4-benzoylbenzoyl)adenosine 5'-triphosphate (BzATP) is frequently used to assess P2X7 receptor-dependent signaling because it activates P2X7 receptors with greater potency than ATP [2]. However, BzATP also activates other subtypes of P2X [2–4] and P2Y receptors [5, 6].

ATP-induced changes in cytosolic pH (pH_i) have been reported in a number of cell types [7–12]. However, not all cell types display changes in pH_i when stimulated with ATP [13], indicating that this is not a universal phenomenon. Osteoblast-like MC3T3-E1 cells express several types of P2X and P2Y receptors, including P2X7 [14, 15]. We have shown previously that the activation of P2X7 receptors on these cells triggers a sustained increase in metabolic acid production [16]; however, no information was available

Juan Pablo Reyes and Matthew W. Grol contributed equally to this work.

J. P. Reyes · S. M. Sims · S. J. Dixon (✉)
Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, The University of Western Ontario, London, Ontario N6A 5C1, Canada
e-mail: jeff.dixon@schulich.uwo.ca

M. W. Grol
Department of Anatomy and Cell Biology, Schulich School of Medicine and Dentistry, The University of Western Ontario, London, Ontario, Canada

Present Address:

J. P. Reyes
Laboratory of Molecular and Cellular Neurobiology, Neurobiology Institute, National Autonomous University of Mexico, Campus UNAM Juriquilla, Juriquilla, Mexico

regarding the effects of extracellular nucleotides on pH_i of MC3T3-E1 cells.

Commercial suppliers provide BzATP as a triethylammonium (TEA) salt. In the present study, we investigated the effects of BzATP-TEA salt on pH_i in MC3T3-E1 cells. BzATP-TEA elicited fast-onset alkalinization responses, but they were not reproduced by a high concentration of ATP. Thus, these responses are unlikely to be mediated by P2 receptors. At physiological pH , solutions of BzATP-TEA salt contain both protonated (TEA^+) and unprotonated (TEA) forms of triethylamine. Diffusion of TEA into cells would be expected to result in cytosolic alkalinization. Using multiple approaches, we found that BzATP-TEA-induced changes in pH_i were mediated by TEA rather than by the activation of P2 receptors. pH_i influences the activity of many cellular processes, including vesicle trafficking, metabolism, cytoskeletal remodeling, and signaling via Ca^{2+} and adenosine 3',5'-cyclic monophosphate [17]. Consequently, when using BzATP-TEA as an agonist to probe the function of P2X7 receptors, it is critical to perform control experiments to distinguish between specific effects that are mediated by P2 receptors and nonspecific effects that are mediated by the actions of TEA on pH_i .

Materials and methods

Cells and culture

The MC3T3-E1 osteoblast-like cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). This is a clonal nontransformed cell line established from newborn mouse calvariae [18]. These cells endogenously express a number of P2 receptor subtypes, including P2X7 [19]. Cells were cultivated in α -minimum essential medium (α -MEM) supplemented with 10 % heat-inactivated fetal bovine serum and 1 % antibiotic–antimycotic solution (all reagents from Invitrogen, Burlington, ON, Canada) in a humidified atmosphere containing 5 % CO_2 at 37 °C. Cells were detached from culture vessels by treatment with 0.05 % trypsin–EDTA solution (Invitrogen) and were passaged twice weekly.

Measurement of cytosolic pH

Semi-confluent MC3T3-E1 cultures were loaded with the pH-sensitive fluorescent dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) by incubation in BCECF acetoxymethyl ester (BCECF-AM, 2 $\mu\text{g}/\text{ml}$ in culture medium; Invitrogen) for 30 min [20]. Cells were then suspended by trypsinization. Experiments were carried out with cells suspended in a cuvette ($\sim 1 \times 10^6$ cells in 2 ml)

with continuous stirring at room temperature. A cuvette-based spectrofluorimeter equipped with a DeltaRam VTM fluorescence excitation system (Photon Technology International, Birmingham, NJ, USA) was used to measure the emission intensity (at 535 nm) when BCECF was alternately excited at 495 nm and at its isosbestic point of 439 nm. The ratio of emission intensities at 495/439 nm excitation provides a measure of pH_i . The extracellular buffer used for these experiments contained (in millimolar): *N*-methyl-D-glucamine chloride, 140; MgCl_2 , 1; CaCl_2 , 1; glucose, 10; and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 20. pH was adjusted to 7.4 with HCl. Nominally Na^+ -free buffer was used to minimize Na^+/H^+ exchange, which can mask changes in pH_i [21]. ATP (disodium salt), BzATP-TEA, and TEA chloride were from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of test substances or vehicle were added directly to the cuvette (pH of all stock solutions was adjusted to 7.4). Note that BzATP-TEA contains ~ 3 TEA ions per molecule of BzATP. Therefore, when TEA chloride was used to assess nonspecific effects of BzATP-TEA, TEA chloride was tested at three times the molar concentration of BzATP-TEA.

Measurement of proton efflux

MC3T3-E1 cells were seeded on porous polycarbonate membranes (Transwell, 12-mm diameter, 3- μm pore size; Corning Inc. Costar, Corning, NY, USA) in supplemented α -MEM at a density of 12×10^4 cells/ cm^2 . After 48 h, polycarbonate membranes with adherent cells were placed in microflow chambers positioned above silicon-based potentiometric sensors, which detect changes in extracellular pH (pH_o) of as little as 10^{-3} units (Cytosensor microphysiometer; Molecular Devices, Sunnyvale, CA, USA) [22]. Cells were continuously superfused at 100 $\mu\text{l}/\text{min}$ with medium at 37 °C. Superfusion medium was bicarbonate-free MEM (Invitrogen) lightly buffered with HEPES (1 mM) and adjusted to $\text{pH } 7.15 \pm 0.02$ with NaOH. Each chamber was supplied with medium from one of two reservoirs selected by a computer-controlled valve. Where indicated, samples were superfused with medium containing BzATP-TEA or TEA chloride, and changes in proton efflux were monitored. In some experiments, medium contained the specific P2X7 antagonist A-438079 (Tocris Bioscience, Bristol, UK). The lag time between a valve switch and the arrival of test solutions at the microflow chambers was 4–5 s.

The surface potential of each silicon sensor, corresponding to the pH_o , was plotted as a voltage–time trace. At 37 °C, 61 mV corresponds to 1 pH unit. To measure the rate of extracellular acidification, fluid flow to cells was stopped periodically for 30 s. During this time, acid accumulated in the microflow chamber (volume, 2.8 μl), causing pH_o to decrease. Measurement of acidification

rate was obtained by linear least squares fit to the slope of the pH_i –time trace during the time when fluid flow to the cells was stopped [23]. Because of an artifact arising from the changing medium, the first data point after superfusion with agonist began was sometimes omitted from the trace.

Measurement of cytosolic free Ca^{2+} concentration

For experiments using the Ca^{2+} -sensitive dye fura-2, MC3T3-E1 cultures were loaded by incubation with fura-2-AM (2 $\mu\text{g}/\text{ml}$ in culture medium; Invitrogen) for 30 min. Cells were then suspended by trypsinization. Experiments were carried out with cells suspended in a cuvette ($\sim 1 \times 10^6$ cells in 2 ml) with continuous stirring at room temperature. A cuvette-based spectrofluorimeter equipped with a DeltaRam VTM fluorescence excitation system (Photon Technology International) was used to measure the emission intensity (at 510 nm) when fura-2 was excited at alternating 340/380-nm wavelengths. The ratio of emission intensities at 340/380 nm excitation provides a measure of cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). The nominally Na^+ -free buffer described previously was used.

For experiments using the Ca^{2+} -sensitive dye indo-1, MC3T3-E1 cultures were loaded by incubation with indo-1-AM (2 $\mu\text{g}/\text{ml}$ in culture medium; Invitrogen) for 30 min. Cells were then suspended by trypsinization. Experiments were carried out as described above. Samples were excited at 355 nm with emission wavelengths recorded at 405 and 485 nm. The 405/485-nm ratio of emission intensities provides a measure of $[\text{Ca}^{2+}]_i$. In experiments using indo-1, cells were suspended in HEPES buffer containing (in millimolar): NaCl, 135; KCl, 5; MgCl_2 , 1; CaCl_2 , 1; glucose, 10; and HEPES, 20. pH was adjusted to 7.3 with NaOH. Stock solutions of BzATP-TEA, TEA chloride, or vehicle were added directly to the cuvette via an injection port.

Statistical analyses

Proton efflux was normalized as a percentage of basal efflux in standard superfusion medium before addition of the test substance. This normalization compensated for differences in cell numbers among the chambers. The amplitude of changes in pH_i or $[\text{Ca}^{2+}]_i$ induced by test substances was quantified as the difference either between baseline and peak or between baseline and sustained phase (defined as the response 10 min posttreatment). Results are presented as the means $\pm$ standard error of the mean (SEM). Differences between groups were evaluated by unpaired Student's *t* test and accepted as statistically significant at $p < 0.05$.

Results and discussion

We studied changes in pH_i elicited by BzATP-TEA, using the pH-sensitive dye BCECF. The application of BzATP-TEA (0.3 or 1.5 mM, final concentrations in the cuvette) elicited fast-onset alkalinization that recovered over time (Fig. 1a). Note that 0.3 mM BzATP-TEA did not saturate the response, since significantly greater amplitude was observed with 1.5 mM BzATP-TEA (Fig. 1b). Thus, it is unlikely that these responses were mediated by P2X7 receptors because they are thought to be saturated at 0.3 mM BzATP [4]. However, the involvement of other P2 receptors with lower affinity for BzATP could not be ruled out. To examine this possibility, we stimulated cells with ATP (the disodium salt, which does not contain TEA). ATP (5 mM, a concentration sufficient to activate P2X7, as well as several other P2 receptors) failed to induce a response similar to that elicited by BzATP-TEA (Fig. 2), suggesting that BzATP-TEA-induced effects were independent of P2 receptor signaling.

Fig. 1 BzATP-TEA induces alkalinization of the cytosol. MC3T3-E1 cells were loaded with the pH-sensitive fluorescent dye BCECF and suspended in nominally Na^+ -free HEPES buffer in a fluorometric cuvette with continuous stirring. Changes in pH_i were monitored by fluorescence spectrophotometry, with alternating excitation at 495 and 439 nm and emission at 535 nm. The ratio of emission intensities at 495/439 nm excitation provides a measure of pH_i , with increasing values reflecting cytosolic alkalinization. **a** Where indicated by the arrows, 0.3 or 1.5 mM BzATP-TEA was added to the cuvette. Traces are representative responses. **b** Changes in pH_i were quantified as the peak amplitude of the response above baseline (baseline values were comparable among preparations). * $p < 0.05$, significant difference between responses to the two BzATP-TEA concentrations. Data are presented as the means $\pm$ SEM ($n = 5$ or 6 independent preparations for 0.3 and 1.5 mM BzATP-TEA, respectively)

Fig. 2 Cytosolic alkalinization induced by BzATP-TEA is independent of P2X7 receptor activation. MC3T3-E1 cells were loaded with BCECF, suspended in Na^+ -free HEPES buffer, and changes in pH_i were monitored by fluorescence spectrophotometry. **a** Where indicated by the arrows, ATP disodium salt (5 mM) or BzATP-TEA (0.3 mM) was added to the cuvette. Traces are representative responses. **b** Changes in pH_i were quantified as the peak amplitude of the response above baseline. * $p < 0.05$, significant difference between responses to 5 mM ATP and 0.3 mM BzATP-TEA. Data are presented as the means $\pm$ SEM ($n = 4$ independent preparations for both ATP and BzATP-TEA)

In the extracellular medium, protonated triethylamine (TEA^+) exists in equilibrium with its unprotonated form (TEA). In the unprotonated form, extracellular TEA can permeate the plasma membrane and be protonated in the cytosol, yielding TEA^+ and increasing pH_i [24] (Fig. 3). Thus, we hypothesized that the effects of BzATP-TEA on pH_i could be mediated by TEA, rather than BzATP. To test this hypothesis, we first treated cells with comparable concentrations of BzATP-TEA and TEA chloride (Fig. 4). Responses to 4.5 mM TEA chloride closely resembled responses to 1.5 mM BzATP-TEA.

Second, we monitored the net transport of protons (and proton equivalents) across the plasma membrane using microphysiometry [22, 23]. Basal proton efflux in standard superfusion medium remained steady for periods of at least 1 h. Superfusion of cells with TEA chloride (3 mM) induced a transient decrease in proton efflux (Fig. 5), consistent with its ability to induce transient alkalinization of the cytosol (Figs. 3 and 4). Moreover, washout of TEA chloride triggered a large transient increase in proton efflux (Fig. 5), consistent with the transient acidification of the cytosol expected upon withdrawal of TEA (Fig. 3).

We have recently reported the actions of BzATP-TEA on proton efflux from MC3T3-E1 cells [16]. BzATP-TEA induced

Fig. 3 Schematic illustrating permeation and protonation of the weak base triethylamine (TEA). **a** When in the extracellular fluid, protonated TEA^+ is in equilibrium with uncharged TEA, which can permeate the plasma membrane. Once in the cytosol, TEA becomes protonated, increasing pH_i . An increase in pH_i leads to a decrease in efflux of protons and proton equivalents via Na^+/H^+ exchange and other pathways. **b** Upon withdrawal of TEA from the extracellular fluid, uncharged TEA leaves the cell. Protons then dissociate from cytosolic TEA^+ , decreasing pH_i . A decrease in pH_i leads to the activation of proton efflux pathways such as Na^+/H^+ exchange. In both cases, the change in proton efflux is transient, as it occurs only until pH_i is restored to its resting level

a sustained increase in metabolic acid production that was dependent on glucose and phosphatidylinositol 3-kinase activity. This sustained increase developed slowly, reaching a maximum at 10–20 min following the application of BzATP-TEA

Fig. 4 TEA chloride elicits changes in pH_i similar to that induced by BzATP-TEA. MC3T3-E1 cells were loaded with BCECF, suspended in Na^+ -free HEPES buffer, and changes in pH_i were monitored by fluorescence spectrophotometry. **a** Where indicated by the arrows, BzATP-TEA (1.5 mM) or TEA chloride (4.5 mM) was added to the cuvette. Traces are representative responses. **b** Changes in pH_i were quantified as the peak amplitude of the response above baseline. Data are presented as the means $\pm$ SEM, $p > 0.05$ ($n = 5$ independent preparations for both BzATP-TEA and TEA)

Fig. 5 TEA chloride elicits transient changes in proton efflux. MC3T3-E1 cells were cultured on porous polycarbonate membranes, and proton efflux was monitored by microphysiometry. Cells were superfused with standard medium, and at 1 min intervals, superfusion was interrupted for 30 s to measure acidification rate. Net efflux of proton equivalents (proton efflux) was calculated from the acidification rate and expressed as a percentage of basal proton efflux. Where indicated by the shaded rectangle, MC3T3-E1 cells were superfused with TEA chloride (3 mM) (closed symbols) or vehicle (open symbols) in standard medium for 12 min. Exposure to TEA promptly induced a transient decrease in proton efflux from 100 to 28 ± 26 % of basal levels. On the other hand, withdrawal of TEA induced a large transient increase in proton efflux to values of 42 ± 49 %. In contrast to the dramatic response to TEA, superfusion with vehicle had no effect. Data are presented as the means $\pm$ SEM ($n=11$ samples from three independent preparations)

[16]. To differentiate between the P2X7-dependent and P2X7-independent effects of BzATP-TEA on proton efflux in the present study, we applied BzATP-TEA in the presence or absence of A-438079, a specific antagonist of the P2X7 receptor [25]. Consistent with our previous report [16], BzATP-TEA (300 μ M) alone caused a large sustained increase in proton efflux that persisted for at least 60 min (Fig. 6). A-438079 (10 μ M) abolished the sustained phase of the BzATP-TEA-induced response (Fig. 6). Notably, exposure to BzATP-TEA in the presence of A-438079 caused a prompt transient decrease in proton efflux, followed by a large transient increase upon wash-out (Fig. 6), comparable to the changes in proton efflux observed in response to TEA chloride alone (Fig. 5). Taken together, these results establish that transient changes in proton efflux elicited by BzATP-TEA are due to receptor-independent effects of TEA on pH_i , whereas the sustained increase in proton efflux elicited by BzATP-TEA is mediated by activation of P2X7 receptors.

The microphysiometry experiments in the present study were performed using medium that was nominally HCO_3^- -free (to avoid the production of gas bubbles) and that contained physiological concentration of Na^+ (116 mM NaCl). Under these conditions, the primary pathway for the efflux of protons (or proton equivalents) in osteoblastic cells is Na^+/H^+ exchange, mediated by sodium/hydrogen exchanger 1 (NHE1) [26, 27]. Na^+/H^+ exchangers are ubiquitously expressed membrane transporters that regulate intracellular pH by removing a proton from the cytosol in exchange for an extracellular

Fig. 6 BzATP-TEA causes a sustained P2X7-dependent increase in proton efflux. MC3T3-E1 cells were cultured on porous polycarbonate membranes and superfused with standard medium. Superfusion was interrupted for 30 s at 1.5 min intervals to measure acidification rate. Where indicated by the horizontal bar beneath the graph, parallel samples were superfused with solution containing either the P2X7 antagonist A-438079 (10 μ M) or control (H_2O). After 6 min, cells were stimulated with either BzATP-TEA (300 μ M) (closed symbols) or vehicle (open symbols) where indicated by the shaded rectangle in the continued presence of the appropriate medium. In control samples, BzATP-TEA caused a large sustained increase in proton efflux that persisted for at least 60 min. In contrast, no sustained phase was apparent in cultures treated with BzATP-TEA in the presence of A-438079. However, exposure to BzATP-TEA in the presence of A-438079 still induced a transient decrease in proton efflux and withdrawal of BzATP-TEA elicited a large transient increase in proton efflux. Note that the pattern of these changes in proton efflux in the presence of the P2X7 receptor antagonist is similar to that observed in response to TEA chloride alone (compare right panel of Fig. 6 to Fig. 5). Data are presented as the means $\pm$ SEM ($n=5-7$ samples from three to four independent preparations)

sodium ion. Moreover, NHE1 activity is regulated by pH_i , with cytosolic acidification increasing NHE1 activity, which then returns pH_i to resting values [28]. Thus, the transient decrease in proton efflux upon exposure to TEA (Fig. 5) likely reflects suppression of NHE activity, whereas the transient increase in proton efflux upon withdrawal of TEA likely reflects enhanced NHE activity. As expected, these changes in proton efflux were transient, since they should only last until pH_i is restored to resting values.

Taken together, our fluorimetry and microphysiometry studies reveal marked effects of TEA on pH_i and proton efflux at concentrations equivalent to those of BzATP-TEA used to activate P2X7 receptors. Therefore, when using BzATP-TEA, effects mediated by TEA-induced changes in pH_i may be mistaken for effects mediated by P2 receptors. Of course, this is particularly relevant when studying the effects of P2X7 activation on proton transport and pH_i . However, this may also apply to the many other cellular processes influenced by pH_i , which include metabolism, motility, and signaling [17].

Given the P2 receptor-independent effects identified in the present study, we suggest that suitable control experiments using TEA chloride (at three times the molar

concentration of BzATP-TEA) be employed whenever working with BzATP-TEA. As an example, we used this approach to investigate the mechanisms underlying the action of BzATP-TEA on $[Ca^{2+}]_i$ in MC3T3-E1 cells. It is known that stimulation of P2 receptors in MC3T3-E1 cells leads to an increase in $[Ca^{2+}]_i$ [16, 29, 30]. Moreover, it has been reported that pH_i influences $[Ca^{2+}]_i$ in these cells [31]. Thus, we investigated whether the Ca^{2+} responses elicited by BzATP-TEA in MC3T3-E1 cells might be secondary to receptor-independent effects of TEA. We first assessed the effects of TEA chloride on Ca^{2+} signaling (Fig. 7). As expected, BzATP-TEA (1 mM) elicited an elevation of $[Ca^{2+}]_i$. In contrast, TEA chloride (3 mM) did not alter $[Ca^{2+}]_i$ (Fig. 7), consistent with the specific effects of BzATP mediated by the activation of P2 receptors.

We next assessed the contribution of P2X7 to the elevation of $[Ca^{2+}]_i$ induced by BzATP-TEA. MC3T3-E1 cells were treated with BzATP-TEA in the presence or absence of A-438079 (Fig. 8). BzATP-TEA (300 μ M) alone elicited a biphasic increase in $[Ca^{2+}]_i$, consisting of an initial transient followed by a sustained elevation (Fig. 8). In the presence of

Fig. 7 BzATP-TEA, but not TEA chloride, induces the elevation of $[Ca^{2+}]_i$. MC3T3-E1 cells were loaded with the Ca^{2+} -sensitive fluorescent dye fura-2 and suspended in Na^+ -free, Ca^{2+} -containing HEPES buffer in a fluorometric cuvette. Changes in $[Ca^{2+}]_i$ were monitored by fluorescence spectrophotometry, with alternating excitation wavelengths of 340 and 380 nm and emission at 510 nm. The ratio of emission intensities at 340/380 nm excitation provides a measure of $[Ca^{2+}]_i$. **a** Where indicated by the arrows, BzATP-TEA (1 mM) or TEA chloride (3 mM) was added to the cuvette. Traces are representative responses. **b** Changes in $[Ca^{2+}]_i$ were quantified as the peak amplitude of the response above baseline. * $p < 0.05$, significant difference between responses to BzATP-TEA and TEA chloride. Data are presented as the means $\pm$ SEM ($n = 5$ and 7 independent preparations for BzATP-TEA and TEA chloride, respectively)

Fig. 8 BzATP elicits a sustained P2X7-dependent elevation of $[Ca^{2+}]_i$. MC3T3-E1 cells were loaded with the Ca^{2+} -sensitive fluorescent dye indo-1 and suspended in Ca^{2+} -containing HEPES buffer in a fluorometric cuvette. Changes in $[Ca^{2+}]_i$ were monitored by fluorescence spectrophotometry, with a 355-nm excitation wavelength, and emission recorded at 405 and 485 nm. The ratio of emission intensities at 405/485 nm provides a measure of $[Ca^{2+}]_i$. **a** BzATP-TEA (300 μ M) caused a rapid rise of $[Ca^{2+}]_i$, with an initial peak followed by a sustained phase. The P2X7 antagonist A-438079 (10 μ M) specifically suppressed the sustained phase, without affecting the initial transient elevation of $[Ca^{2+}]_i$. Traces are representative responses from four independent preparations. **b** Changes in $[Ca^{2+}]_i$ were quantified as the peak amplitude of the response above baseline. **c** Changes in $[Ca^{2+}]_i$ were also quantified as the amplitude of the sustained phase of the response above baseline, determined at 10 min following the addition of BzATP-TEA. * $p < 0.05$, significant effect of A-438079. Data are presented as the means $\pm$ SEM ($n = 4$ independent preparations)

A-438079 (10 μ M), BzATP-TEA induced only the initial Ca^{2+} transient. Thus, BzATP-TEA elicits the elevation of $[Ca^{2+}]_i$ in MC3T3-E1 cells through interaction with multiple P2 receptor subtypes. The sustained phase is due to the activation of P2X7 receptors, whereas the initial transient is due to the activation of other Ca^{2+} -mobilizing P2 receptors present in these cells. In this regard, it is well established that BzATP can activate P2 receptors other than P2X7 [2–6]. In this example, the control experiment with TEA chloride revealed that the effects of BzATP-TEA on $[Ca^{2+}]_i$ were not secondary to TEA-mediated changes in pH_i (Fig. 7). Moreover, the control experiment with A-438079 revealed that the BzATP-induced elevation of $[Ca^{2+}]_i$ arises by the activation of multiple P2 receptor subtypes (Fig. 8).

In conclusion, these studies reveal that TEA, present in the commonly used BzATP salt, induces nonspecific effects unrelated to P2 receptor signaling. We suggest that investigators using BzATP-TEA perform control experiments using TEA chloride to avoid potential misinterpretation of their findings.

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