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Two-phase response of acid extrusion triggered by purinoceptor in Chinese hamster ovary cells

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Abstract

The functional characteristics of purinoceptors in Chinese hamster ovary (CHO) cells were investigated using a microphysiometer which detects small metabolic changes to living cells in real-time as variations of pH in the extracellular microenvironment. Uridine 5′-triphosphate (UTP) increased the extracellular acidification rate biphasically, namely a transient and a steady response were observed. The transient phase reached a peak (four- to fivefold the basal extracellular acidification rate in amplitude) within 20 s and was followed by the steady phase which was sustained for more than 1 min at an amplitude less than twofold the basal extracellular acidification rate. Both phases showed a concentration-dependent increase in response to UTP. However, there was a significant difference in the pEC_{50} value for UTP between the transient (4.8) and steady phases (6.1). Like UTP, ATP increased the extracellular acidification rate, but α,β-methyleneATP (α,β-MeATP), 2-methylthioATP (2-MeSATP), ADP, UDP and adenosine did not. This result suggests that the acid is extruded through a $P2Y_2$ or $P2Y_2$-like purinoceptor. 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) and 4-isopropyl-3-methylsulphonylbenzoyl-guanidine methanesulphonate (HOE642) suppressed both phases of the UTP-stimulated extracellular acidification rate response with high affinity (pIC_{50}: approximately 7.0). This result suggests that the Na^+/H^+ exchanger 1 (NHE-1) predominantly mediates the UTP-induced acid extrusion response in CHO cells. Elimination of extracellular Ca^{2+} or treatment with thapsigargin diminished both phases of the UTP-stimulated extracellular acidification rate. In addition, *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride (W-7) also abrogated the two phases. These results are consistent with the involvement of NHE-1 which is activated via Ca^{2+}/calmodulin. Persistent exposure to UTP reduced both extracellular acidification rate phases, causing desensitization of the P2Y purinoceptor. This desensitization did not affect the acid extrusion response mediated by the α_1-adrenoceptor.

Keywords: Acid extrusion; Microphysiometer; P2Y purinoceptor; Na^+/H^+ exchanger

1. Introduction

Extracellular pH was believed to be maintained within a narrow range at around 7.4 by homeostatic mechanisms. However, recent investigations have revealed that the extracellular as well as intracellular pH is not as constant as generally thought but under dynamic regulation by various factors such as cell metabolism and ionic membrane transport (Chesler, 1990; Deitmer and Rose, 1996). Extracellular pH changes are often estimated using pH-sensitive microelectrodes and magnetic resonance, but such methods have drawbacks, in particular, a poor quantitative evaluation and/or low time resolution (Chesler, 1990; Smith et al., 1998). Recently, Taniguchi et al. (2001) have demonstrated that extracellular acidification is induced in seconds while using a microphysiometer.

The microphysiometer is an instrument to measure extracellular pH and to assess the extracellular acidification rate which reflects both the metabolic state of a cell through the extrusion of acidic metabolites and the homeostasis of the intracellular pH through the regulation of proton transport across the cytoplasmic membrane (McConnell et al., 1992; Owicki et al., 1990). Extracellular purines and pyrimidines are important signaling molecules that mediate a range of biological events including smooth muscle contraction, neurotransmission, exocrine and endocrine secretion, immune responses, inflammation, platelet aggregation, nociception and the modulation of cardiac function (Ralevic

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and Burnstock, 1991, 1998; Dubyak and el-Moatassim, 1993). The purines and pyrimidines act on two main families of purinoceptors, the P1 and P2 receptors, which are expressed in a wide variety of cells and tissues. In this study, the role of purinoceptors in acid extrusion was investigated using Chinese hamster ovary (CHO) cells and a microphysiometer. The present study clearly shows that the purinoceptor triggers the extrusion of acid from CHO cells.

2. Materials and methods

2.1. Materials

Alpha minimum essential medium, fetal bovine serum and G418 were obtained from Gibco BRL (Grand Island, NY); 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) and thapsigargin from Research Biochemicals International (Natick, MA); *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride (W-7) from Calbiochem (Darmstadt, Germany); ADP, UDP and EGTA from Nacalai tesque (Kyoto, Japan); adenosine from Kohjin (Tokyo, Japan); ATP, α,β-methyleneATP (α,β-MeATP), 2-methylthioATP (2-MeSATP), uridine 5′-triphosphate (UTP) and noradrenaline from Sigma (St Louis, MO, USA); and 4-isopropyl-3-methylsulphonylbenzoyl-guanidine methanesulphonate (HOE642) from Aventis Pharma (Tokyo, Japan).

2.2. Cell culture

A cell line of CHO cells, aH5, which stably expresses human α_{1A}-adrenoceptor (Taniguchi et al., 1999) was cultured in alpha minimum essential medium containing 10% fetal bovine serum and 200 μg ml^{-1} of G418 at 37 °C in a humidified atmosphere of 5% CO_2/95% O_2.

2.3. Measurement of the extracellular acidification rate

The extracellular acidification rate was measured using a microphysiometer as previously described (Taniguchi et al., 2001). Briefly, the aH5 cells (3×10^5) were seeded into the microphysiometer cup, 24 h prior to the experiment. On the commencement of the experiment, the cup was loaded into the microphysiometer chamber. In order to detect small amounts of acid extruded from the cells, a low-buffered balanced salt solution (LBS) was used as the running medium: the composition being 130 mM NaCl, 1.5 mM $CaCl_2$, 3 mM KCl, 0.6 mM $MgCl_2$, 10 mM glucose, 0.2 mM KH_2PO_4 and 0.8 mM Na_2HPO_4, 5 mg l^{-1} phenol red, pH 7.4. Fig. 1A shows the raw potentiometric data of the microphysiometer recordings which reflect the changes of extracellular pH; when the pump was off, the pH of LBS in the chamber gradually reduced due to the extrusion of acid from the cells appearing as a downward deflection (Fig. 1A, bold line), while upon

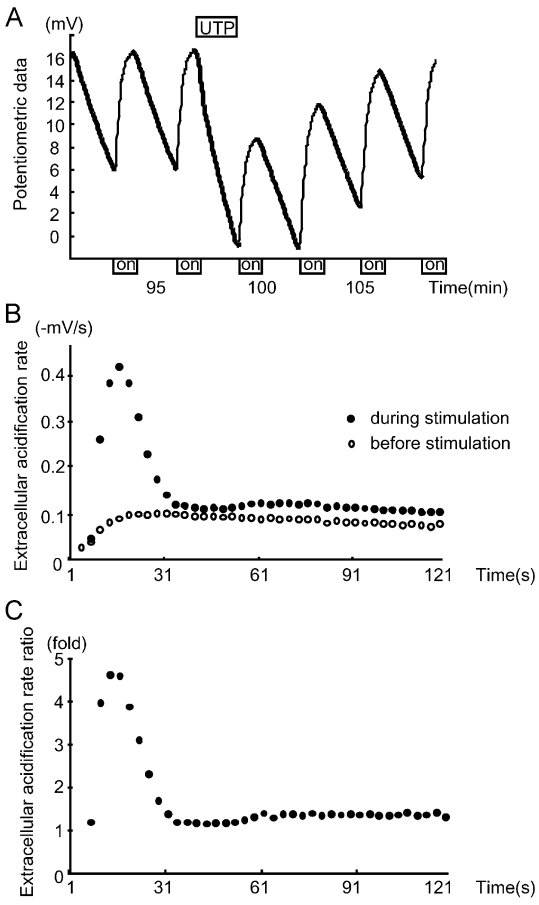

Fig. 1. Real-time assessment of extracellular acidification. (A) A representative potentiometric recording of extracellular pH is shown in mV. Boxes in the abscissa indicate "flow on" for 60 s. Between the boxes, perfusion was stopped for 120 s, during which the change in extracellular pH was measured (bold line). After basal acid extrusion became constant, the CHO cells were stimulated with 30 μM UTP as described under Materials and methods. (B) Absolute values of extracellular acidification rate (− mV/s). Extracellular acidification rates every 3 s during 120-s "flow off" were plotted before and during UTP application. (C) Extracellular acidification rate ratio was estimated by dividing the extracellular acidification rate during the application of UTP by the basal extracellular acidification rate (before the application) at the corresponding time point during "flow off". UTP elicited a rapid and transient increase in extracellular acidification rate that was followed by a steady phase.

perfusion, the pH rose reaching that of LBS (Fig. 1A, thin line). Perfusion speed was 100 μl min^{-1} and a 180-s pump cycle was used: "flow on" for 60 s and "flow off" for 120 s. This means that the acid extrusion was measured as the acidification rate of the chamber medium during the cessation of perfusion for 120 s. Under these experimental conditions, it has been reported that a change of 6.1 mV in the potentiometer corresponds to a change of pH 0.1 (McConnell et al., 1992). Once the basal acidification rate (a downward deflection during "flow off") was stable and reproducible, the cells were stimulated with 30 μM UTP three times at 30-min intervals, and the third response to UTP was reproducible and taken as a standard in the same

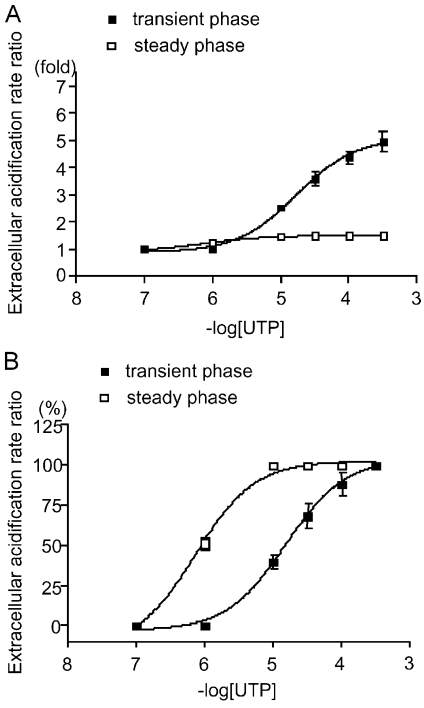

Fig. 2. Concentration response curves of extracellular acidification rate for UTP. As shown in Fig. 1C, the transient phase was measured at the peak of the extracellular acidification rate ratio but the steady phase was measured as the mean extracellular acidification rate ratio between 71 and 110 s. (A) Absolute extracellular acidification rate ratios of transient and steady phases. (B) Extracellular acidification rate ratios of transient and steady phases were respectively normalized against the maximum responses produced by 0.3 mM UTP. Data represent the mean ± S.E.M. from eight independent experiments.

chamber. Then, increasing concentrations of UTP (0.1–300 μM) were added for 121 s again at 30-min intervals to establish a concentration–response relationship, as mentioned elsewhere. In order to detect an early response, the cells were exposed to UTP 1 s before and continuously during "flow off", resulting in the real-time monitoring of extracellular acidification from 1 to 121 s after the application. In inhibitor experiments, a control response to UTP was recorded without the inhibitor, then the cells were perfused with LBS containing an inhibitor 15 min prior to and throughout the subsequent responses to UTP (see Fig. 5A). The pH of a medium which include UTP or other drugs were adjusted at 7.4 just before use.

2.4. Data analysis

Acid extrusion was analysed as the extracellular acidification rate. That is, extracellular acidification rate was estimated from a slope every 3 s in the potentiometric recording during "flow off" and the absolute values (−mV/s) were first plotted as shown in Fig. 1B. However, since the extracellular acidification rate varied among the microphysiometer chambers used, extracellular acidification rates before and during UTP treatment were compared at the same time points during "flow off" and the ratios were plotted as the extracellular acidification rate ratio (Fig. 1C). EC50 values were calculated, using PRISM (GraphPad Software, San Diego, CA, USA). Data are presented as the mean ± standard error mean (S.E.M.).

3. Results

3.1. Effect of UTP on acid extrusion

Upon the cessation of perfusion, the extracellular pH in the chamber gradually decreased and was detected as a downward deflection in the potentiometric recording (Fig. 1A, bold line). The acidification before drug application reflects the basal acid extrusion from the cells. The acidification was significantly accelerated during exposure to 30 μM UTP, resulting in a faster and larger downward deflection as compared with the basal acidification before UTP. Extracellular acidification rate during 120 s "flow off" was measured as a slope every 3 s and the time courses before and during UTP application were plotted in Fig. 1B. To estimate the acceleration of acid extrusion, we furthermore compared the extracellular acidification rate

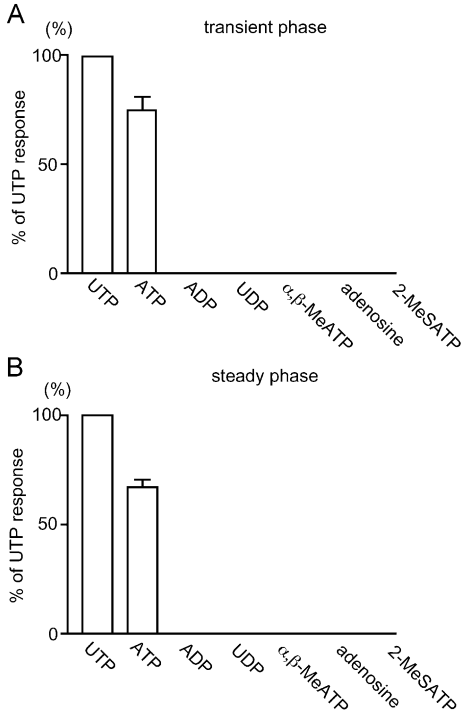

Fig. 3. Effects of various purinoceptor agonists on acid extrusion. The CHO cells were stimulated with 30 μM of UTP, ATP, ADP, UDP, α,β-MeATP, adenosine or 2-MeSATP, and transient and steady phases of the extracellular acidification rate were compared with those produced by 30 μM UTP in the same experiment. Data represent the mean ± S.E.M. from three to eight independent experiments.

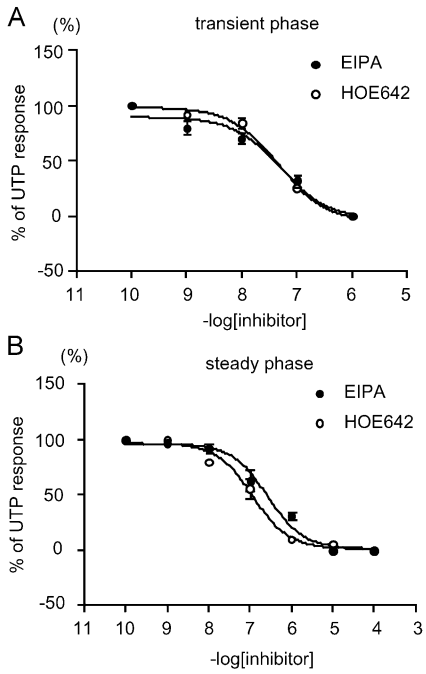

Fig. 4. Effect of NHE inhibitors on the acid extrusion response. Acid extrusion produced by submaximum concentrations of UTP (A: 100 μM for transient phase; B: 10 μM for steady phase) was compared before and after treatment with various concentrations of EIPA (close symbols) or HOE642 (open symbols). Data represent the mean ± S.E.M. from three independent experiments.

during UTP application with the basal extracellular acidification rate before the application. Therefore, Fig. 1C shows the extracellular acidification rate ratio against the basal extracellular acidification rate. As shown in Fig. 1B and C, UTP elicited a rapid and transient acid extrusion that was followed by a smaller steady phase. The transient phase reached a peak within 20 s of the application of UTP and the steady phase was maintained from 60 to 120 s.

Fig. 2 shows the concentration–response curves of the transient and steady phases of extracellular acidification rate for UTP. The amplitude of the transient phase was greater than that of the steady phase (Fig. 2A). When the two phases were respectively normalized against the maximum responses to 0.3 mM UTP (Fig. 2B), both had distinct pEC_{50} values for UTP (4.8 and 6.1 for the transient and steady phase, respectively).

3.2. Effects of various purinoceptor agonists on acid extrusion

The effects of ATP, ADP, UDP, α,β-MeATP, adenosine and 2-MeSATP on acid extrusion were tested at a concentration of 30 μM and each extracellular acidification rate response was compared with that to 30 μM UTP (a standard response in the same experiment). As shown in Fig. 3, ATP at 30 μM elicited about a 70% response relative to UTP in both the transient and steady phase. However, the other drugs tested did not increase the extracellular acidification rate.

3.3. Pharmacological characterization of UTP-induced acid extrusion

The distinct pEC_{50} values for the transient and steady phases of the extracellular acidification rate suggested that the two might be elicited by different mechanisms. First the effects of two Na^+/H^+ exchanger (NHE) inhibitors, EIPA and HOE642, were examined. Both produced a concentration-dependent inhibition of the extracellular acidification rate response to UTP without changing the basal extracellular acidification rate (Fig. 4), indicating that NHE was predominantly involved in the UTP-stimulated acid extrusion. pIC_{50} values for EIPA and HOE642 were both 7.3 in the transient phase, and 6.6 and 6.9, respectively, in the steady phase.

Next, the signal transduction pathways involved in the acid extrusion response to UTP were investigated. Elimination of extracellular Ca^{2+} diminished both the transient and steady phases of extracellular acidification rate (Fig. 5B). Replenishment of Ca^{2+} restored the biphasic response (data not shown). Thapsigargin at 3 μM also abrogated the two phases (Fig. 5C). W-7 at 70 μM suppressed both the transient and steady phases of extracellular acidification rate stimulated by UTP (Fig. 5D).

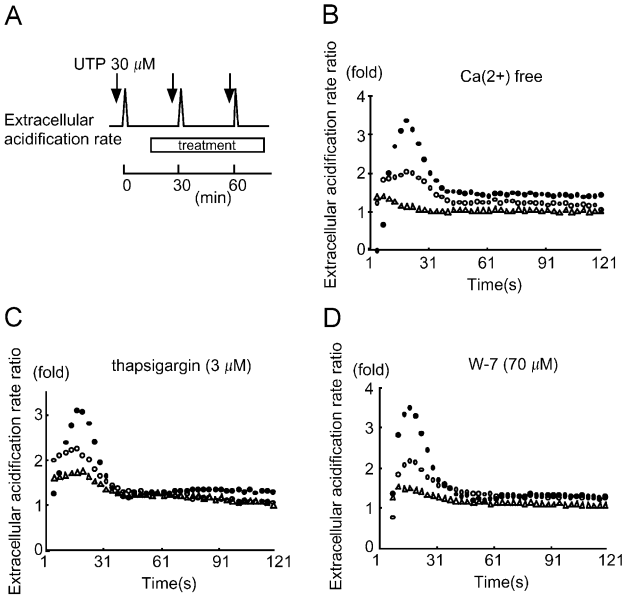

Fig. 5. Effects of various treatments on the acid extrusion induced by UTP in CHO cells. (A) Experimental protocol. UTP at 30 μM was applied for 120 s every 30 min as shown by the arrows. After the first response to UTP was recorded (closed circles), various treatments were applied, and the second (open circles) and third responses (open triangles) were recorded. (B) Effect of Ca^{2+} removal. Ca^{2+}-free LBS medium with 0.3 mM EGTA was perfused. (C) Effect of thapsigargin (3 μM). (D) Effect of W-7 (70 μM). The data are representative of three to four experiments in each case.

3.4. Desensitization and resensitization of UTP-induced acid extrusion

To examine whether desensitization exists in the UTP-induced acid extrusion, the CHO cells were continuously exposed to 30 μM UTP for 2 h. Thereafter, the cells were perfused with UTP-free LBS medium and the recovery of the UTP response was examined. This protocol is outlined in Fig. 6A. As described above, UTP initially produced a biphasic extracellular acidification rate response to UTP (Fig. 6, a in A and closed circles in B). The subsequent treatment with UTP for 2 h abrogated the response (Fig. 6, b and c in A, and open circles in B and closed circles in C). Thereafter, the response to UTP gradually recovered during 3 h of washing (Fig. 6C). The steady phase was more rapidly restored than the transient phase.

Fig. 6D shows the effects of ATP (30 μM) and noradrenaline (30 μM) in the cells which had been exposed to 30 μM UTP for 2 h. Noradrenaline produced an increase in extracellular acidification rate but ATP did not.

These desensitizations were not observed in short time exposures used in Figs. 1–5 (data not shown).

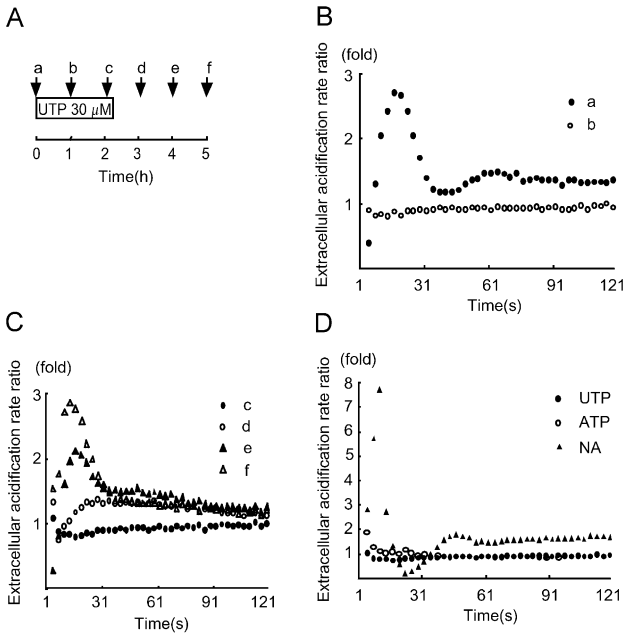

Fig. 6. Effects of sustained exposure to UTP on acid extrusion in CHO cells. (A) Experimental protocol. The cells were exposed to 30 μM UTP for 2 h and thereafter perfused with UTP-free LBS medium for 3 h. The acid extrusion response to 30 μM UTP was measured every hour (a–f). (B) Extracellular acidification rate ratio at the commencement (a) of and 1 h (b) into UTP exposure. (C) Recovery of acid extrusion response to UTP after sustained treatment with UTP. Note the fast recovery of the steady phase as compared with the transient phase. (D) Effects of ATP or noradrenaline in the cells exposed to 30 μM UTP for 2 h. At arrow c shown in the protocol of part A, the cells were stimulated with 30 μM ATP (open circles) or 30 μM noradrenaline (closed triangles) instead of 30 μM UTP (closed circles). The same results were obtained in four experiments with each agonist.

4. Discussion

4.1. Two-phase acid extrusion response via purinoceptor

The microphysiometer has been used to detect changes of cell activity upon the stimulation of a variety of receptors (Fischer et al., 1999; McConnell et al., 1992; Owicki et al., 1990). The authors developed a new measurement method that allowed real-time monitoring of extracellular acidification rate in the microphysiometer (see Sections 2 and 3) and previously demonstrated an α_{1A}-adrenoceptor-driven rapid acid extrusion in CHO cells (Taniguchi et al., 2001). In the present study also, UTP elicited a significant response in the form of the extrusion of acid from CHO cells, which was composed of two phases, transient and steady. Purinoceptor-mediated acid extrusion was previously reported in cholangiocytes or human colonic adenocarcinoma cells (Elsing et al., 1996; Richards et al., 1997), but the biphasic response was first observed with the real-time monitoring of the present study. The transient phase was approximately five-fold the peak of the basal extracellular acidification rate, indicating that acid extrusion is dramatically and rapidly (in seconds) produced by the application of UTP in CHO cells.

4.2. Responses by various purinoceptor agonists

As mentioned above, the extracellular acidification rate was composed of two phases. Since the amplitude of extracellular acidification rate and the pEC_{50} value for UTP differed between these phases, it was first examined whether both phases are mediated through the same type of receptors or not. In addition to UTP, ATP also elicited a biphasic extracellular acidification rate albeit the amplitudes were slightly smaller than those for UTP. In contrast, neither α,β-MeATP, 2-MeSATP, ADP, UDP nor adenosine accelerated the extrusion (Fig. 3). It has been reported that CHO cells express the P2U purinoceptor (Iredale and Hill, 1993), which corresponds to the $P2Y_2$ purinoceptor in recent nomenclature (Ralevic and Burnstock, 1998). The selectivity of the agonist used in the present study suggests that a single purinoceptor, the $P2Y_2$ or a $P2Y_2$-like receptor, is involved in both the transient and steady phases of acid extrusion in response to UTP.

4.3. Pharmacological characterization of UTP-induced acid extrusion

Both phases of the extracellular acidification rate accelerated by UTP were inhibited by NHE inhibitors, EIPA and HOE642, with the same potency (pIC_{50}: approximately 7.0). NHE constitutes a family of six members, among which the ubiquitous expression of NHE-1 has been demonstrated in mammalian cells including CHO cells (Counillon and Pouyssegur, 2000; Clark and Limbird, 1991). Among NHEs, NHE-1 is highly sensitive to EIPA (Yu et al., 1993) and HOE642 (Scholz et al., 1995). Therefore, it

was speculated that NHE-1 mediates both phases of acid extrusion upon stimulation of the purinoceptor in CHO cells. This is in part different from our previous report in which α_{1A}-adrenoceptor also drove a two-phase extrusion system in CHO cells; the transient phase was mediated by NHE-1, while the steady phase was mediated by not only NHE-1 but unknown mechanisms with low affinity for both EIPA and HOE642 (Taniguchi et al., 2001). This may imply that different receptors can couple to a distinct set of acid transport systems although a major component of acid extrusion is mediated by NHE-1.

Since the $P2Y_2$ purinoceptor triggers the recruitment of intracellular Ca^{2+} as one of the initial intracellular signaling events (Erb et al., 1993; Iredale and Hill, 1993), the possible relationship between extracellular acidification rate and Ca^{2+} was examined. Elimination of extracellular Ca^{2+} diminished the transient and steady phases of the UTP-stimulated extracellular acidification rate. Both phases of acid extrusion were also inhibited by thapsigargin, an intracellular Ca^{2+} depleter (Thastrup et al., 1990), and by W-7, a calmodulin antagonist (Tanaka and Hidaka, 1980). Since NHE-1 activity is regulated by Ca^{2+} and calmodulin (Betrand et al., 1994; Wakabayashi et al., 1994), the present results suggest that a purinoceptor–Ca^{2+}–NHE-1 pathway is involved in the acid extrusion induced by UTP.

4.4. Desensitization and resensitization of purinoceptor-induced acid extrusion

Prolonged exposure to UTP caused not only the transient but also the steady phase of extracellular acidification rate to subside in CHO cells, suggesting that the purinoceptor and/or subsequent processes undergo desensitization (Fig. 6B). After successive exposure to UTP, ATP also did not evoke an increase in extracellular acidification rate (Fig. 6D). However, the stimulation of α_{1A}-adrenoceptor expressed in CHO cells caused an increase in extracellular acidification rate irrespective of successive exposure to UTP (Fig. 6D). As well as the $P2Y_2$ purinoceptor, α_1-adrenoceptor is known to cause the release of Ca^{2+} from intracellular stores (Garcia-Sainz et al., 2000; Suzuki et al., 2000). These results suggest that the loss of the UTP-induced effect is due to desensitization of the purinoceptor itself, not to inactivation of the subsequent intracellular signaling pathways (Garrad et al., 1998; Otero et al., 2000). They also indicate no heterologous desensitization between the purinoceptor and α_{1A}-adrenoceptor.

After desensitization, the response to UTP was gradually restored under UTP-free conditions (Fig. 6C). Here, it is interesting that the recovery was faster in the steady than transient phase. Since the pEC_{50} value for UTP was higher in the steady phase (6.1) than in the transient phase (4.8), smaller amounts of purinoceptors may be needed to recover the steady phase than the transient phase.

4.5. Conclusions

In conclusion, the present study demonstrates the existence of a receptor-operated rapid acid extrusion process in CHO cells, which is caused by a Ca^{2+}-dependent activation of NHE-1. What is the physiological significance of this response? Generally, the buffering capacity of the extracellular space and net movement of acid/base equivalents determine extracellular pH. The buffering capacity of the extracellular fluid is based on its buffer value and the volume of the fluid in the extracellular space. The former is around 50 mM in terrestrial animals (Chesler, 1990; Deitmer and Rose, 1996) and the latter is approximately 10^{-7} μl/cell in humans: for example, a human 70 kg in body weight has approximately 10^{14} cells and 10 l of extracellular fluid, resulting in an ample volume of extracellular fluid per single cell at 10^{-7} μl. Buffering capacity is, thus, 5 fmol/cell in humans. In the case of microphysiometry, the buffer is 1 mM phosphate with an approximately 0.5 mM buffer value and the space for a cellular response in the chamber is a few microliters (McConnell et al., 1992) for 3×10^5 cells. Therefore, the buffering capacity in microphysiometry will also be approximately 5 fmol/cell. Thus, it is possible to deduce that rapid acidification in response to purine might happen in a limited local space in vivo as seen in the microphysiometer, acting as a paracrine and/or autocrine signal in the microenvironment.

Acknowledgements

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