Dual Effect of Acid pH on Purinergic P2X<sub>3</sub> Receptors Depends on the Histidine 206 Residue*

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Received for publication, July 16, 2007, and in revised form, September 11, 2007 Published, JBC Papers in Press, September 21, 2007, DOI 10.1074/jbc.M705840200

Whole cell patch clamp investigations were carried out to clarify the pH sensitivity of native and recombinant P2X<sub>3</sub> receptors. In HEK293 cells permanently transfected with human (h) P2X<sub>3</sub> receptors (HEK293-hP2X<sub>3</sub> cells), an acidic pH shifted the concentration-response curve for α,β-methylene ATP (α,β-meATP) to the right and increased its maximum. An alkalic pH did not alter the effect of α,β-meATP. Further, a low pH value increased the activation time constant (τ<sub>on</sub>) of the α,β-meATP current; the fast and slow time constants of desensitization (τ<sub>des1</sub>, τ<sub>des2</sub>) were at the same time also increased. Finally, acidification accelerated the recovery of P2X<sub>3</sub> receptors from the desensitized state. Replacement of histidine 206, but not histidine 45, by alanine abolished the pH-induced effects on hP2X<sub>3</sub> receptors transiently expressed in HEK293 cells. Changes in the intracellular pH had no effect on the amplitude or time course of the α,β-meATP currents. The voltage sensitivity and reversal potential of the currents activated by α,β-meATP were unaffected by extracellular acidification. Similar effects were observed in a subpopulation of rat dorsal root ganglion neurons expressing homomeric P2X<sub>3</sub> receptor channels. It is suggested that acidification may have a dual effect on P2X<sub>3</sub> channels, by decreasing the current amplitude at low agonist concentrations (because of a decrease in the rate of activation) and accelerating both the kinetics of channel opening and the rate of desensitization. Thereby, a differential regulation of pain sensation during e.g. inflammation may occur at the C fiber terminals of small DRG neurons in peripheral tissues.

High proton concentrations have been registered in inflamed tissue (down to pH 5.4), after surgical interventions (down to pH 5.5), in fracture-related hematomas (down to pH 4.7), in cardiac ischemia (down to pH 5.7), and in and around malignant tumors (1–5). Therefore, local acidosis is considered to contribute to pain experienced in these states (5–11). It is also known that continuous administration of low pH buffered solutions into human skin evokes instant pain and hyperalgesia to mechanical stimulation (12). Electrophysiological experiments in rat skin nerve preparations showed that pathophysiologically relevant high proton concentrations produce a selective non-adapting excitation of nociceptors and a significant sensitization to mechanical stimulation (13). Thus, it has been proposed that local acidosis may play a major role in pain and hyperalgesia (7).

Hydrogen ions are able to excite dorsal root ganglion (DRG) neurons via the activation and/or modulation of inward cation-selective currents, including the acid-sensing ion channels (ASICs) (14), the transient receptor potential vanilloid receptor 1 (TRPV1) (15, 16), and P2X receptors (17, 18). P2X receptors represent a family of ligand-gated cationic channels that open in response to the binding of ATP, possess two transmembrane domains, intracellular N and C termini, a large extracellular loop, and assemble as homo- or heterotrimers (19–21). In peripheral tissues, large quantities of ATP may leave the intracellular space in response to tissue trauma, tumors, inflammation, migraine, or visceral distension (22). The resulting P2X receptor activation and the subsequent depolarization of the sensory cell membrane initiate action potentials that are perceived centrally as pain. Although sensory neurons express all known P2X subunits, the homomeric P2X<sub>2</sub> and the heteromeric P2X<sub>2,3</sub> receptors occur in these cells at the highest density (23, 24).

Whereas agonist-induced currents through recombinant P2X<sub>2</sub> and P2X<sub>2,3</sub> receptor channels (17, 18) were potentiated by acidification, the same change in pH depressed currents through P2X<sub>3</sub> receptor channels (18). The failure to use more than a single concentration of α,β-meATP did not allow to construct concentration-response relationships for the P2X<sub>3</sub> receptor subunit. Bullfrog DRG and rat nodose ganglion neurons are known to possess native P2X<sub>1</sub>, P2X<sub>2</sub>, and P2X<sub>3</sub> receptors. Application of ATP onto these cells evoked non-desensitizing currents resembling P2X<sub>2</sub> or P2X<sub>2,3</sub> receptor activation (19). These currents were enhanced by acidification, suggesting that extracellular protons can regulate the function of ATP-gated receptor channels in bullfrog DRG and rat nodose ganglion neurons (25, 26).

The aim of the present study was to investigate the effect of acidification on recombinant human P2X<sub>3</sub> receptors (hP2X<sub>3</sub>R) transfected into human embryonic kidney (HEK) 293 cells. It was found that a change of pH from the normal 7.4 to 5.8 markedly decelerated both the kinetics of channel opening and the

* This work was supported by the Deutsche Forschungsgemeinschaft (IL 20/11-3). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: DRG, dorsal root ganglion; ASIC, acid-sensing ion channel; α,β-meATP, α,β-methylene ATP; WT, wild type.
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subsequent speed of desensitization, and thereby depressed currents caused by low, but facilitated currents caused by high agonist concentrations. Our findings demonstrate a dual, agonist concentration-dependent effect of acid pH at homomeric P2X₃ receptors which may allow a differential modulation of pain sensation in response to small or large quantities of ATP released by noxious stimulation.

MATERIALS AND METHODS

Culturing of HEK293-hP2X₃ Cells—The maintenance of HEK293 cells and their stable transfection with hP2X₃R cDNA have been described previously (27). Cells were kept in Dulbecco’s modified Eagle’s medium also containing 25 mM HEPES, 110 μg/ml sodium pyruvate, 1 mg/ml d-glucose, 4 μg/ml pyridoxine (Invitrogen, Karlsruhe, Germany), 2 mM L-glutamine, 1% non-essential amino acids (NEAA) (allSigma), 10% fetal bovine serum and 50 μg/ml geneticin (both from Invitrogen) at 37 °C and 10% CO₂ in humidified air. They were plated on 35-mm plastic dishes (Sarstedt, Nürnberg, Germany) for electrophysiological recordings.

Site-directed Mutagenesis and Transfection Procedures—The human P2X₃ receptor cDNA (GenBank™ accession number NM-00255) was subcloned per PstI and EcoRI restriction sites into pIRE2-EGFP vector from Clontech Laboratories (Mountain View, CA) for independent expression of P2X₃ and EGFP, creating the pIR-P2 plasmid. All P2X₃ receptor mutants were generated by introducing point mutations into the pIR-P2 construct, using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA) according to the instruction manual. HEK293 cells were plated in 35-mm plastic dishes 1 day before transient transfection. 0.5 μg of plasmid DNA per dish was combined with 10 μl of Polyfect reagent from Qiagen (Hilden, Germany) and 100 μl of OptiMEM (Invitrogen, Karlsruhe, Germany). After 10 min of incubation, the lipid-DNA complexes were introduced to the cells. Approximately 18-h post-transfection, the medium was replaced with OptiMEM to remove residual Plasmid DNA. Electrophysiological recordings took place 48–72 h after transient transfection during maximum protein expression. P2X₃ receptor-positive cells were detected by viewing EGFP fluorescence with a fluorescence microscope.

Preparation of DRG Neuronal Cultures—One-day-old Wistar rats (own breed) were used in the study. The animals were killed under CO₂ and decapitated to obtain cell cultures of thoracic and lumbar DRG cells. The isolation and culturing procedures of DRG neurons. The isolation and culturing procedures of DRG neurons have been described in detail previously (28, 29). DRG cells were plated at a density of 3 x 10⁵ cells onto 35-mm plastic dishes coated with poly-l-lysine (25 μg/ml) (Sarstedt). They were kept in Dulbecco’s modified Eagle’s medium, 35 mM total glucose, 2.5 mM L-glutamine, 15 mM HEPES, 50 μg/ml gentamicin, 5% fetal bovine serum (Invitrogen), 30 ng/ml nerve growth factor, 10 μg/ml insulin, 5.5 μg/ml transferrin, and 5 ng/ml selenium (Sigma). Primary cultures of rat DRG neurons were maintained for 2–4 days in a humidified atmosphere (37 °C, 5% CO₂) before experimentation.

Whole Cell Patch Clamp Recordings—Whole cell patch-clamp recordings were performed 2–6 days after the splitting of permanently transfected HEK293 cells, and 2–4 days after the plating of rat DRG neurons, at room temperature (20–22 °C), using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Union City, CA). Patch pipettes (3–5 MΩ) for both HEK293 cells and DRG neurons were filled with intracellular solution of the following composition (in mM): 135 CsCl, 2 MgCl₂, 20 HEPES, 11 EGTA, 1 CaCl₂, 1.5 Mg-ATP, and 0.3 Li-GTP. pH was adjusted with CsOH. The external solution consisted of (in mM) 140 NaCl, 5 KCl, 2 MgCl₂, 2 CaCl₂, 10 HEPES, and 11 glucose, pH was adjusted with NaOH. The pH of all solutions was routinely checked before and during experiments. All recordings were made at a holding potential of ~70 mV. Data were filtered at 2 kHz with the in-built filter of the Axopatch 200B, digitized at 5 kHz, and stored on a laboratory computer using a Digidata 1200 interface and pClamp 10.0 software (Molecular Devices).

Drugs were dissolved in external solution and applied locally to single cells, using a rapid solution change system (SF-77B Perfusion Fast-Step, Warner Instruments, Hamden, CT; 10–90% rise time of the junction potential at an open pipette tip was 1–4 ms). Concentration-response curves for the P2X₃ receptor currents at different pH values were constructed by applying every 5 min increasing concentrations of α,β-methyl-ATP (α,β-meATP). To analyze P2X₃ currents from the same cells at different pH values, α,β-meATP was applied at a given concentration six times with 5-min intervals. After the recording of two α,β-meATP-induced inward currents of the same size at pH 7.4, the pH was changed 2.5 min after the second α,β-meATP administration. Two α,β-meATP currents were recorded at the altered pH value and two further ones at pH 7.4 again. When recording from DRG cells, amiloride (200 μM) was given to the bath solution to block ASICs. This concentration of amiloride did not affect P2X₃ currents either in HEK293-hP2X₃ cells or in DRG neurons (see “Results”). Concentration-response curves established to determine the agonistic effects of α,β-meATP were fitted to mean data points using Equation 1,

\[ I = I_{max} \times \frac{[A]^{nH}}{([A]^{nH} + EC_{50}^{nH})} \]  

(Eq. 1)
activation and desensitization was found to be concentration-independent and was expressed as the mean ± S.E. of the time constant ratios at each concentration investigated. In experiments examining the recovery of P2X3 receptors from desensitization, HEK293 cells were stimulated repetitively with α,β-meATP at 3 μM (5-s pulses, each) with a progressive increase in the interpulse intervals. The recovery from desensitization was fitted using the following Equation 3 (30),

\[ I = I_{\text{max}} + \frac{I_{\text{max}} - I_{\text{min}}}{1 + e^{-t/t_{\text{50}}}} \]  

(Eq. 3)

where \( I_{\text{max}} \) and \( I_{\text{min}} \) are the start and finish levels during recovery, \( t \) is the time, \( t_{\text{50}} \) is the time to regain 50% of maximally recovered currents, and \( b \) is the slope factor giving the change in time per e-fold change in recovery.

Materials and Drugs—α,β-Methylene ATP lithium salt (α,β-meATP) and amiloride hydrochloride were both purchased from Sigma. The drugs were prepared as a concentrated stock solution in distilled water and were diluted to the final concentration in external medium.

Data Analysis—Data were analyzed off-line using pClamp 10.0 software (Molecular Devices). Figures show mean ± S.E. values of \( n \) experiments. Student’s \( t \) test or one way ANOVA followed by Bonferroni’s post hoc test were used for statistical analysis. A probability level of 0.05 or less was considered to reflect a statistically significant difference.

RESULTS

Effect of Extracellular Protons on P2X3 Currents in HEK293-hP2X3 Cells—Application of various concentrations of α,β-meATP to HEK293 cells permanently transfected with hP2X3Rs (HEK293-hP2X3 cells) induced inward current responses (Fig. 1A). Acidification of the extracellular solution had 3-fold effects on the concentration-response curves for α,β-meATP. It enhanced the maximal current amplitudes (\( I_{\text{max}} \)), increased the EC\(_{50}\) values, and raised the Hill coefficient (\( nH \); Fig. 1Ba and Table 1). In contrast, the alkalization of the extracellular solution caused no major change in the \( I_{\text{max}} \), the EC\(_{50}\) value or the \( nH \) (Fig. 1Bb; Table 1).

To further investigate the effect of protons on P2X3 receptors, we applied two selected agonist concentrations (1 and 4.64 μM) onto HEK293-hP2X3 cells and examined, whether a change in the pH affects P2X3 currents in the same cell. As shown in Fig. 2, the current amplitudes evoked by α,β-meATP at 1 μM (approximate EC\(_{50}\)) were inhibited by shifting the pH from 7.4 to 5.8 by 43.7 ± 8.6% \((p < 0.05)\) whereas at 4.64 μM (high concentration of this agonist), the current amplitudes were facilitated by 28.9 ± 14.0% \((p < 0.05)\) (Fig. 2A). Both the increase and the decrease of the current responses to these α,β-meATP concentrations were reversible on returning to the original extracellular pH of 7.4. In contrast, a change of the pH from 7.4 to 8.0 did not modify the α,β-meATP currents at either concentration (1.4 ± 6.3%, \( n = 6 \) and 1.8 ± 8.6%, \( n = 6 \), at 1 and 4.64 μM, respectively) (Fig. 2B).

The original tracings in Fig. 2, A and B show that acidification, but not alkalization itself induces rapidly declining inward currents. These currents may be due to the activation of ASICs described to be present in HEK293 cells (31). As documented in Fig. 2, Aa and Ab, these currents completely desensitized within milliseconds, and therefore they are not expected to alter the currents evoked by α,β-meATP 2.5-min later.

**FIGURE 1.** Effects of changes in the extracellular pH on currents through human P2X3 receptors permanently transfected into HEK293 cells (HEK293-hP2X3 cells). A, representative tracings of P2X3 currents evoked by α,β-meATP at three levels of pH (AA, 7.4; AB, 8.0; AC, 5.8). The horizontal lines above the recordings indicate the superfusion times. The concentrations are in μM. Note that gray lines designate the low α,β-meATP concentrations, whereas black lines designate the high α,β-meATP concentrations. B, concentration-response curves for P2X3 currents evoked by α,β-meATP at acidic (Ba) and alkalic (Bb) pH values, when compared with the normal pH value of 7.4. The mean ± S.E. of 7–14 experiments is shown.

| pH   | \( I_{\text{max}} \) | \( nH \) | EC\(_{50}\) |
|------|-----------------|---------|----------|
| 7.4  | 1.38 ± 0.29     | 1.13 ± 0.29 | ND*      |
| 7.8  | 1.54 ± 0.05     | 0.66 ± 0.02 | 1.19 ± 0.05 |
| 8.0  | 1.75 ± 0.24     | 0.77 ± 0.08 | 1.01 ± 0.07 |
| 5.8  | 1.55 ± 0.49     | 1.74 ± 0.58 | 0.74 ± 0.17 |
| 5.4  | 2.88 ± 0.09*    | 1.67 ± 0.02* | 0.42 ± 0.03* |

*ND not determined.

**TABLE 1** Parameters of the α,β-meATP concentration-response relations and the \( t_{\text{50}} \) values of the recovery from α,β-meATP-induced desensitization for human P2X3 receptors permanently transfected into HEK293 cells at various pH levels.

The parameters were determined from the concentration-response curves in Fig. 1B and from the recovery curves in Fig. 4B by means of equations described under “Materials and Methods.” The mean ± S.E. of 7–14 experiments is shown.

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Nonetheless, to reliably exclude a possible interference of ASIC currents with α,β-meATP-induced currents, the same experiments as shown in Fig. 2A were performed in the presence of amiloride, a selective inhibitor of ASIC channels (14). In fact, amiloride (200 μM) abolished the early current induced by a change of pH from 7.4 to 5.8 (96.5 ± 2.7% inhibition, n = 5, p < 0.05%), but had no effect on the response to α,β-meATP (1 μM) at a pH of 5.8 (40.3 ± 9.6% inhibition, n = 5, p > 0.05%; compare with Fig. 2Ae). It is noteworthy that the lower concentration (1 μM) of α,β-meATP evoked a current response at a pH of 5.8, which desensitized much slower than that observed at the control pH value of 7.4 (Fig. 2Aa; see below).

Effect of Protons on P2X3 Receptor Activation and Desensitization—Next, we investigated whether different proton concentrations affect the activation and desensitization of P2X3 channels. The current decay in the presence of α,β-meATP, which represents the onset of desensitization, was fitted by a biexponential equation (see "Materials and Methods") and had a first fast and a second slow time constant (τdes1, τdes2). Of these time constants, τdes1 was found to be the more dominant one, because it constituted concentration-independently 84.9 ± 1.4% of the current decay, as calculated from the contribution of the relative amplitudes of the first and second exponential to the total current amplitude (A1/A1 + A2, see Equation 2 under "Materials and Methods"; n = 39). The activation time constants (τon) and both desensitization time constants (τdes1, τdes2) inversely correlated with the α,β-meATP concentration (Fig. 3, Ca–Cc). These data are in accordance with earlier observations showing that the kinetics of activation, desensitization, and recovery from desensitization, but not deactivation of P2X receptors, inversely correlate with the agonist concentration (32–34).

Lowering the pH to 5.8 increased both the activation time constant and the two desensitization time constants at all ago-
nist concentrations applied (Fig. 3, A, B, and Ca–Cc). Increasing the pH value up to 8.6 did not change the activation and desensitization time constants appreciably. The normalized shift of the pH value up to 8.6 did not change the activation and desensitization time constants appreciably. The normalized shift of the pH value up to 8.6 did not change the activation and desensitization time constants appreciably. The normalized shift of the pH value up to 8.6 did not change the activation and desensitization time constants appreciably.

It is interesting to note that the concentration versus $t_{on}$ curves were less steep than the concentration versus $t_{des1}$ curves. Thus at lower concentrations of $\alpha$, $\beta$-meATP the current amplitude was determined mostly by the activation time constant, whereas at higher concentrations of $\alpha$, $\beta$-meATP the current amplitude was determined mostly by the first time constant of desensitization (Fig. 3, Ca and Cc).

Effect of Protons on the Recovery from Desensitization—After rapid desensitization, P2X$_3$ receptors stay in the desensitized state for minutes and cannot fully open in response to further agonist application. We next examined the availability of the receptors after different time intervals i.e. how fast they recover from this desensitized state and, moreover, whether changing the proton concentration modulates this process. HEK293-hP2X$_3$ cells were stimulated repetitively with $\alpha$, $\beta$-meATP (10 $\mu$M, for 5 s), by progressively increasing the interpulse intervals (Fig. 4). During the application of $\alpha$, $\beta$-meATP, P2X$_3$ receptors completely desensitized. The recovery of P2X$_3$ receptors from desensitization exhibited a sigmoidal time course and was best fitted with Equation 3 described under "Materials and Methods." The time course of recovery was expressed as $t_{up}$, namely the time to regain 50% of the maximally recovered current amplitudes. Lowering the pH to 5.8 significantly accelerated the recovery (Table 1; analysis of variance, $p < 0.05$, $n = 7–8$), whereas increasing the pH to 8.0 had no effect on the speed of recovery (Table 1).

The Role of Histidine Residues in pH Sensitivity—Protons modulated P2X$_3$ receptors in the range between pH 7.4 and 5.8. The only amino acid with a pK$_a$ close to this range is histidine. The pK$_a$ of free histidine is 6.0 (35), but in proteins its pK$_a$ can range from 5.0 to 8.0 (36) making histidines apparent candidates for the proton-binding site. To test whether histidines are involved in the modulation of P2X$_3$ receptors by protons, we substituted single histidine residues by alanines in the extracellular domain of the human P2X$_3$ receptor (H45A and H206A).

The wild-type (WT) P2X$_3$ receptors transiently transfected into HEK293 cells responded to acidification in a manner similar to that reported for the permanently transfected receptor (compare Tables 1 and 2). In fact, lowering the pH from 7.4 to 5.8 shifted the concentration-response curve for $\alpha$, $\beta$-meATP to the right and increased the $I_{max}$ (Fig. 5, Aa and Ab). We investigated the effect of replacing two histidine residues in the extracellular loop of the P2X$_3$ receptor by the uncharged amino acid alanine (H45A, H206A) on the pH sensitivity of the $\alpha$, $\beta$-meATP effect (Fig. 5B and Table 2). The most important finding is that the H206A mutation abolished the increase of the maximal response, the shift of the concentration-response curve to the right, and the changes in all three kinetic parameters ($t_{des1}$, $t_{des2}$, and $t_{on}$) by acidification to a pH of 5.8 (Fig. 5, Ba and C). In contrast, the single mutation H45A, accentuated the pH-sensitivity of the receptor; both the increase of the maximal response and the rightward shift of the concentration-response curve were more pronounced than in the case of the

![FIGURE 4. pH-dependent recovery from desensitization of hP2X$_3$ receptors in HEK293 cells. A, representative currents evoked by repetitive stimulation by 5-s pulses of $\alpha$, $\beta$-meATP (10 $\mu$M) at different pH levels (7.4, 5.8, 8.0), with progressive increase in the interpulse intervals. B, recovery from $\alpha$, $\beta$-meATP-induced desensitization was fitted with a sigmoid curve (for Equation 3 see "Materials and Methods.") Acidification from a pH of 7.4 to 6.5 and then to 5.8 gradually accelerated the recovery.

| Parameter | pH | $I_{max}$ | $n$ | $EC_{50}$ |
|-----------|----|-----------|----|----------|
| WT        | 7.4| 3 399 ± 33 | 1.78 ± 0.09 | 1.89 ± 0.06 |
|           | 5.8| 5 498 ± 220  | 1.71 ± 0.35 | 5.85 ± 1.20  |
| H206A     | 7.4| 3 345 ± 37 | 1.98 ± 0.23 | 3.48 ± 0.24  |
|           | 5.8| 5 150 ± 48 | 1.64 ± 0.22 | 7.24 ± 0.70  |
| H45A      | 7.4| 9 48 ± 40 | 1.94 ± 0.30 | 0.46 ± 0.04  |
|           | 5.8| 1 633 ± 120 | 2.23 ± 0.50 | 4.50 ± 0.57  |
| DM*       | 7.4| 2 42 ± 13  | 1.55 ± 0.31 | 4.38 ± 0.72  |
|           | 5.8| 2 25 ± 4 | 2.20 ± 0.19 | 15.41 ± 0.80  |

$p < 0.05$, statistically significant difference from pH 7.4 (Student’s t-test). $^*$ DM, double mutant (H206A-H45A).
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**FIGURE 5. Site-directed mutagenesis of histidine 206 to alanine (H206A) abolishes the effects of acidification on hP2X₃ receptors.** HEK293 cells were transiently transfected with the hP2X₃ receptor or its mutants. A, concentration-response curves for WT P2X₃ receptor currents evoked by αβ-meATP at pH 7.4 and pH 5.8. The transiently transfected WT P2X₃ receptor responded to acidification in a manner similar to that observed with the permanently transfected receptor (see Fig. 1). The mean ± S.E. of 8–13 experiments is shown. Ab, representative P2X₃ currents from HEK293 cells transfected with the WT P2X₃ receptor. 3 or 30 μM of αβ-meATP was applied at pH 7.4 (left traces) and pH 5.8 (right traces). B, concentration-response curves for mutant P2X₃ receptors (H206A, H45A, and the double mutant DM) evoked by αβ-meATP at pH 7.4 and pH 5.8. The mean ± S.E. of 6–15 experiments is shown. C, normalized shift of current onset, and desensitization rates (τdes1, τdes2) by acidification to pH 5.8 for WT and mutant P2X₃ receptors. The mutation of His206 to Ala and the double mutation (DM: H206A/H45A) abolished the effect of pH 5.8 on the maximum current. In addition, the mutation H206A abolished the rightward shift of the concentration-response curve and the double mutation H206A/H45A decreased the magnitude of the shift of the curve observed for the single mutation H45A.

**FIGURE 6. Protons affect P2X₃ receptors from the extra- to the intracellular side and do not alter the ion selectivity of the receptor-channel in HEK293-hP2X₃ cells.** Aa, hP2X₃ currents evoked by αβ-meATP (1 μmol) were inhibited by acidification of the extracellular solution to pH 5.8. Ab, this inhibition did not change, when the intracellular pH was lowered from 7.4 to 5.8. B, inhibition of αβ-meATP (1 μmol)-evoked hP2X₃ receptor current amplitudes by extracellular acidification to pH 5.8 at two intracellular pH levels (5.8 and 7.4). Ca, hP2X₃ currents evoked by αβ-meATP (10 μmol) over a range of holding potentials (−80 to +40 mV with 20-mV increments) at an extracellular pH of 7.4. D, current-voltage relationships showing the amplitudes of P2X₃ currents activated by 10 μmol αβ-meATP as a function of membrane potential, at an extracellular pH of 7.4 (open circles) and pH 5.8 (filled circles). The mean ± S.E. of 5–6 experiments is shown. Acidification to pH 5.8 did not alter the reversal potential of hP2X₃ currents.

at pH 7.4 or 5.8 via the patch-clamp pipette (Fig. 6). The amplitude of currents evoked by αβ-meATP (1 μmol) at an extracellular pH (IC pH 7.4: 1.3 ± 0.2 nA, n = 10; IC pH 5.8: 1.3 ± 0.3 pA, n = 9; p > 0.05) (compare Fig. 6, Aa and Ab). Moreover, the amplitude of the current evoked by 1 μmol αβ-meATP was inhibited by a shift in the pH of the extracellular solution from 7.4 to 5.8 to a similar extent, irrespective of whether the pH of the IC solution was 7.4 or 5.8 (by 69 ± 5 and 74 ± 5%, respectively, p > 0.05; Fig. 6B).

**Voltage Sensitivity**—To test whether protons alter the ion permeation ratio of P2X₃ receptor channels, the effect of protons on the reversal potential of αβ-meATP current was also investigated. 10 μmol of αβ-meATP was applied over a range of holding potentials (−80 to +40 mV, 20 mV increments) at pH 7.4 and pH 5.8. Fig. 6C shows the voltage-current relationship for αβ-meATP-activated currents at different pH values. Changing the pH did not alter the reversal potential of the currents activated by αβ-meATP (1 ± 2 mV at pH 7.4 and −1 ± 2 mV at pH 5.8; p > 0.05; n = 6–7).

**Effect of Extracellular Protons on P2X₃ Currents in DRG Cells**—Application of αβ-meATP to small diameter (20–35 μm) rat DRG neurons evoked inward currents with different kinetic properties. Out of the total number of 42 neurons, 38% responded with rapidly desensitizing currents (fast-type).
Another 28% of the neurons responded with slowly desensitizing currents (slow type) and 34% could be classified into the intermediate type, with two-phase kinetics consisting of consecutive rapidly and slowly desensitizing components. The fast-type neurons were suggested to contain homomeric P2X3 receptors, the slow-type neurons heteromeric P2X2/3 receptors, and the intermediate-type neurons a mixture of P2X3 and P2X2/3 receptors (37–39). Only experiments with fast-type responses to α,β-meATP were evaluated in the experiments below.

Lowering the extracellular pH to 5.8 had two effects on the rapidly desensitizing currents (Fig. 7). First, this procedure significantly increased the EC50 value (2.98 ± 0.44 μM and 27.86 ± 3.95 μM at pH 7.4 and 5.8, respectively, n = 7–9, p < 0.05). Second, acidification significantly enhanced the maximal current amplitudes (I_{max}, 644 ± 21 pA and 952 ± 43, n = 7–9, p < 0.05).

**DISCUSSION**

The present experiments demonstrated that protons are able to modulate homomeric P2X3 receptors, constituting about 40% of the receptor population of rat DRG neurons. Lowering the pH slowed down both the kinetics of channel opening and the rate of desensitization to α,β-meATP, facilitated the recovery from the desensitized state, and had a dual effect on the current amplitudes. Whereas the action of low agonist concentrations was inhibited by protons, that of high agonist concentrations was facilitated. Site-directed mutagenesis of histidine 206 at the extracellular loop of the P2X3 receptor to alanine abolished the acid sensitivity. These results indicate that pH modulation of the hP2X3 receptor is mediated by protonation of H206 and gives further explanation how local acidosis could attenuate or facilitate pain.

H+ ions are known to differentially modulate various P2X receptors. Acidification inhibited P2X3 receptor currents (18) by increasing the EC50 value for ATP without a concomitant change in the maximum ATP effect (40, 41). P2X4 receptor currents were also found to be inhibited by acidification (18, 42). The EC50 value of ATP was uniformly increased under these conditions, although some of the authors reported that the maximum effect of ATP remained constant (18), while other ones found it to become smaller (42). In contrast, protons potentiated both recombinant and native P2X3 receptor currents by reducing the EC50 value without altering the maximum effect of ATP (17, 18, 43).

Bullfrog DRG and rat nodose ganglion neurons are known to express homomeric P2X1, P2X2, and P2X3, as well as heteromeric P2X2/3 receptors (44, 45). Application of ATP onto these neurons evoked non-desensitizing currents which resembled P2X3 or P2X2/3 receptor activation and were enhanced by acidification (25, 26, 46). Lowering the pH reduced the EC50 value, but did not alter the maximum effect of ATP for evoking these currents.

Only little is known about the modulation of homomeric P2X3 receptors by protons. In an early study, recombinant rat homomeric P2X3 receptors expressed in HEK293 cells and activated by a stable concentration of ATP (1 μM) were found to be depressed by acidification to pH 6.3 (18). In another study, homomeric rat P2X3 receptors expressed in *Xenopus oocytes* were activated by ATP; acidification to pH 5.5 inhibited the current amplitudes. This inhibition was due to a 15-fold increase in the EC50 value for ATP without a concomitant change in the maximum ATP effect (40).

Here, we report that acidification to pH 5.8 caused a 1.6-fold increase in the Hill coefficient and a 2.2-fold increase in the EC50 value for α,β-meATP to activate human homomeric P2X3 receptor channels. Besides these effects we also found that such a change in the extra-, but not intracellular pH considerably increased the maximum α,β-meATP effect (I_{max}). Finally, acidification decreased the desensitization rate of the P2X3 receptor and accelerated the speed of recovery from its desensitized state.

Protons are able to modulate P2X3 currents by a number of possible mechanisms. These include: 1) altering the affinity of the agonist to bind to the receptor, 2) altering the efficacy of the agonist to activate the channel, 3) altering the rate of receptor desensitization, and 4) altering the ion permeation ratio.

First, acidification shifted the concentration-response curve to the right indicating that protons reduce the affinity of the agonist to its receptor. However, the macroscopic EC50 value does not necessarily reflect a change in the microscopic affinity (i.e. the rate constants of association and dissociation) because it depends also on the effects of pH on the gating rate constants (opening, closing, desensitization, and recovery) (47). To answer the question more precisely, concentration dependence should be analyzed on the single channel level. However, the fast single channel kinetics of the receptor (48) makes it difficult to perform a proper analysis.

Second, lowering extracellular pH increased the maximal α,β-meATP-evoked current indicating that protons act by increasing the efficacy of α,β-meATP to activate the P2X3 receptor. Just as an increased EC50 value does not prove a decreased microscopic affinity (see above), an increased apparent efficacy does not reflect accurately how microscopic rates of opening and closing are affected, because apparent efficacy is also determined by (microscopic) agonist affinity, and by desensitization recovery rates.
Dual Effect of Acid pH on P2X<sub>3</sub> Receptors

Third, the decay rate of P2X<sub>3</sub> receptors was clearly decreased by lowered pH, indicating that the channel desensitizes at a slower rate. This may be one of the causes of decreased apparent affinity and increased apparent efficacy. The amplitude of the P2X<sub>3</sub> receptor current is determined by τ<sub>on</sub> and τ<sub>des1</sub> time constants, which both depend on the concentration of the agonist. Because the concentration versus τ<sub>on</sub> curves were less steep in our experiments than the concentration versus τ<sub>des1</sub> curves, the amplitude of the current at low agonist concentrations is primarily determined by τ<sub>on</sub> while at high agonist concentrations primarily by τ<sub>des1</sub>. This may explain that acidification at the same time can depress and facilitate αβ-meATP effects. The inhibition of the response at low agonist concentration may be due to an effect on τ<sub>on</sub> while the facilitation of the response at high concentrations may be due to an effect on τ<sub>des1</sub>. Finally, protons did not alter the reversal potential of currents activated by αβ-meATP, indicating that protons did not affect the ion selectivity of the channel.

All pH-dependent effects were abolished when the histidine 206 of the extracellular loop was mutated to alanine. Histidine residues at the extracellular loop of P2X<sub>2</sub> (H319) and P2X<sub>4</sub> (H286) receptors were found to play a role in the modulation of these subunits (42, 49). His<sup>206</sup> is also located in the extracellular loop somewhat closer to the second transmembrane domain than in P2X<sub>2</sub> or P2X<sub>4</sub>. The pK<sub>α</sub> of free histidine is 6.0 (35) (in proteins its pK<sub>α</sub> can range from 5.0 to 8.0 (36)). Hence, our results suggest that protonation of the histidine residue 206 changes the energy balance of the channel and thereby slows down the transition into both open and desensitized states.

It is interesting to note that a bimodal action of protons on ATP currents has been reported for native P2X<sub>2</sub> receptors in rat PC12 cells (50). These authors found that acidification potentiated the effect of low ATP concentrations and attenuated the effect of high ATP concentrations. They explained these results by a facilitated binding of the agonist to resting as well as open receptors, which causes the potentiation at low concentrations and the increased fading (resulting in depression) at high concentrations. They suggested, but did not prove the role of extra-cellular histidines in this phenomenon. Our data concerning the behavior of P2X<sub>3</sub> receptors are fully compatible with these observations, although the effects observed by us were just of the opposite direction.

In conclusion, we have demonstrated a dual effect of acidification on P2X<sub>3</sub> channels in response to agonist application. The current amplitude was decreased at low agonist concentrations (due to a decrease in the rate of activation) and increased at high agonist concentrations (due to a decrease in the rate of desensitization). A balance between these two opposing influences may determine the function of P2X<sub>3</sub> receptor channels. Hence, the effect of low ATP concentrations may be alleviated during inflammation and the accompanying acidification, whereas the effects of large local concentrations of ATP caused by a massive tissue damage may be potentiated by a decrease of pH. This may result in a differential regulation of pain sensation by the C fiber terminals of small DRG neurons in peripheral tissues.

Acknowledgments—We thank Dr. A. Mike and R. Karoly (Department of Pharmacology, Institute of Experimental Medicine, Budapest, Hungary) for many helpful discussions and H. Sobottka and M. Henschke for expert technical assistance.

REFERENCES

1. Hӓbler, C. (1929) Klin. Wochenschr. 1569–1572
2. Revici, E., Stoopen, E., Frenk, E., and Ravich, R. A. (1949) Bull. Inst. Appl. Biol. 1, 21–38
3. Peer, L. A. (1955) Transplantation of Tissues, The Williams & Wilkins Company, Baltimore, MD
4. Jacobs, W. E., Taylor, G. J., Hollis, D. P., and Nunnally, R. L. (1977) Nature 265, 756–758
5. Woo, Y. C., Park, S. S., Subieta, A. R., and Brennan, T. J. (2004) Anesthesiology 101, 468–475
6. Issberner, U., Reeh, P. W., and Steen, K. H. (1996) Neurosci. Lett. 208, 191–194
7. Reeh, P. W., and Steen, K. H. (1996) Prog. Brain Res. 113, 143–151
8. Sutherland, S. P., Benson, C. J., Adelman, J. P., and McCleskey, E. W. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 711–716
9. Pan, H. L., Longhurst, J. C., Eisenach, J. C., and Chen, S. R. (1999) J. Physiol. 518, 857–866
10. Garber, K. (2003) J. Natl. Cancer Inst. 95, 770–772
11. Stubbs, M., McSheehy, P. M., Griffiths, J. R., and Bashford, C. L. (2000) Mol. Med. Today 6, 15–19
12. Steen, K. H., and Reeh, P. W. (1993) Neurosci. Lett. 154, 113–116
13. Steen, K. H., Reeh, P. W., Anton, F., and Handwerker, H. O. (1992) J. Neurosci. 12, 86–95
14. Wemmie, J. A., Price, M. P., and Welsh, M. J. (2006) Trends Neurosci. 29, 578–586
15. Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D., and Julius, D. (1997) Nature 389, 816–824
16. Tominaga, M., Caterina, M. J., Malmberg, A. B., Rosen, T. A., Gilbert, H., Skinner, K., Raumann, B. E., Basbaum, A. I., and Julius, D. (1998) Neuron 21, 531–543
17. King, B. F., Wildman, S. S., Ziganshina, L. E., Pintor, J., and Burnstock, G. (1997) Br. J. Pharmacol. 121, 1445–1453
18. Stoop, R., Surprenant, A., and North, R. A. (1997) J. Neurophysiol. 78, 1837–1840
19. North, R. A. (2002) Physiol. Rev. 82, 1013–1067
20. Illes, P., and Ribeiro, J. A. (2004) Eur. J. Pharmacol. 483, 5–17
21. Vial, C., Roberts, J. A., and Evans, R. J. (2004) Trends Pharmacol. Sci. 25, 487–493
22. Burnstock, G. (1996) Ciba Found. Symp. 198, 1–28
23. Chizh, B. A., and Illes, P. (2001) Pharmacol. Rev. 53, 553–568
24. North, R. A. (2004) J. Physiol. 554, 301–308
25. Li, C., Peoples, R. W., and Weight, F. F. (1996) J. Neurophysiol. 76, 3048–3058
26. Li, C., Peoples, R. W., and Weight, F. F. (1997) Pflugers Arch. 433, 446–454
27. Gerevich, Z., Zadori, Z., Müller, C., Wirkner, K., Schröder, W., Rubini, P., and Illes, P. (2007) Br. J. Pharmacol. 151, 226–236
28. Gerevich, Z., Bervendeg, S. I., Schröder, W., Franke, H., Wirkner, K., Nörenberg, W., Fürst, S., Gillen, C., and Illes, P. (2004) J. Neurosci. 24, 797–807
29. Gerevich, Z., Müller, C., and Illes, P. (2005) Eur. J. Pharmacol. 521, 34–38
30. Sokolova, E., Skorinkin, A., Fabbretti, E., Masten, L., Nistri, A., and Giniatullin, R. (2004) Br. J. Pharmacol. 141, 1048–1058
31. Lalo, U., Pankratov, Y., North, R. A., and Verkhratsky, A. (2007) J. Cell Physiol. 221, 473–480
32. Zemkova, H., He, M. L., Koshimizu, T. A., and Stoiljkovic, S. S. (2004) J. Neurosci. 24, 6968–6978
33. Sokolova, E., Skorinkin, A., Moiseev, I., Agrachev, A., Nistri, A., and Giniatullin, R. (2006) Mol. Pharmacol. 70, 373–382
34. Yan, Z., Liang, Z., Obsil, T., and Stoiljkovic, S. S. (2006) J. Biol. Chem. 281, 32649–32659
35. Tanokura, M. (1983) Biochim. Biophys. Acta 742, 576–585
36. Kao, Y. H., Fitch, C. A., Bhattacharya, S., Sarkisian, C. J., Lecomte, J. T., and Garcia-Moreno, E. B. (2000) Biophys. J. 79, 1637–1654
37. Burgard, E. C., Niforatos, W., van, B. T., Lynch, K. J., Touma, E., Metzger, R. E., Kowaluk, E. A., and Jarvis, M. F. (1999) J. Neurophysiol. 82, 1590–1598
38. MacKenzie, A. B., Surprenant, A., and North, R. A. (1999) Ann. N. Y. Acad. Sci. 868, 716–729
39. Ma, B., Wynn, G., Dunn, P. M., and Burnstock, G. (2006) Purinergic Signalling 2, 481–489
40. Wildman, S. S., King, B. F., and Burnstock, G. (1999) Br. J. Pharmacol. 128, 486–492
41. Wildman, S. S., King, B. F., and Burnstock, G. (1999) Br. J. Pharmacol. 126, 762–768
42. Clarke, C. E., Benham, C. D., Bridges, A., George, A. R., and Meadows, H. J. (2000) J. Physiol. 523, 697–703
43. Zhong, Y., Dunn, P. M., and Burnstock, G. (2001) Br. J. Pharmacol. 132, 221–233
44. Xiang, Z., Bo, X., and Burnstock, G. (1998) Neurosci. Lett. 256, 105–108
45. Hubscher, C. H., Petruska, J. C., Rau, K. K., and Johnson, R. D. (2001) Neuroreport 12, 2995–2997
46. Li, C., Peoples, R. W., and Weight, F. F. (1996) Neuroreport 7, 2151–2154
47. Colquhoun, D. (1998) Br. J. Pharmacol. 125, 924–947
48. Grote, A., Boldogkoi, Z., Zimmer, A., Steinhauser, C., and Jabs, R. (2005) Mol. Membr. Biol. 22, 497–506
49. Clyne, J. D., LaPointe, L. D., and Hume, R. I. (2002) J. Physiol 539, 347–359
50. Skorinkin, A., Nistri, A., and Giniatullin, R. (2003) J. Gen. Physiol. 122, 33–44