Acidic extracellular pH-activated outwardly rectifying chloride current in mammalian cardiac myocytes

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Yamamoto, Shintaro, and Tsuguhsa Ehara. Acidic extracellular pH-activated outwardly rectifying chloride current in mammalian cardiac myocytes. Am J Physiol Heart Circ Physiol 290: H1905–H1914, 2006.—Extracellular acidic pH was found to induce an outwardly rectifying Cl– current (I_{Cl,acid}) in mouse ventricular cells, with a half-maximal activation at pH 5.9. The current showed the permeability sequence for anions to be SCN– > Br– > I– > Cl– > F– > aspartate, while it exhibited a time-dependent activation at large positive potentials.

Similar currents were also observed in mouse atrial cells and in atrial and ventricular cells from guinea pig. Some Cl– channel blockers (DIDS, niflumic acid, and glibenclamide) inhibited I_{Cl,acid}, whereas tamoxifen had little effect on it. Unlike volume-regulated Cl– current (I_{Cl,vol}) and CFTR Cl– current (I_{Cl,CFTR}), I_{Cl,acid} was independent of the presence of intracellular ATP. Activation of I_{Cl,acid} appeared to be also independent of intracellular Ca2+ and G protein.

I_{Cl,acid} and I_{Cl,vol} could develop in an additive fashion in acidic hypotonic solutions. Isoprenaline-induced Ca2+ and G protein. I_{Cl,acid} and I_{Cl,vol} in HEK293 cells. However, they argued that I_{Cl,acid} and I_{Cl,vol} are different manifestations of the same channel. According to their proposal, acidification alters the properties of the I_{Cl,vol} channels, so that acidification alone can activate a I_{Cl,vol} whose pharmacological and biophysical characteristics are quite different from those of I_{Cl,vol} at normal pHo.

Recently, however, Lambert and Oberwinkler (15) extensively studied the properties of I_{Cl,acid} and I_{Cl,vol} in HEK293 cells. Their important findings are as follows. The recorded single-channel events, which were likely to underlie the whole cell I_{Cl,acid} showed characteristics different from those of I_{Cl,vol} channels recorded in endothelial cells (22). The outward I_{Cl,acid} showed a biphasic change on external acidification, first decreasing and then increasing, and this behavior was difficult to explain by a simple transition model like that predicted by Nobles et al. (19). I_{Cl,acid} and I_{Cl,vol} showed, respectively, time-dependent activation and inactivation during depolarization, and the experiments in which repetitive depolarizing pulses were applied to the cells revealed that the above time-dependent behavior of each current differently persisted even under acidic hypotonic conditions. On the basis of these findings, Lambert and Oberwinkler (15) concluded that I_{Cl,acid} and I_{Cl,vol} are caused by different channels.

As described above, I_{Cl,acid} has been demonstrated in cultured nonexcitable cells. We report here that acidic pHo activates I_{Cl,acid} in freshly isolated mammalian cardiac cells. Our results show that the biophysical and pharmacological properties of cardiac I_{Cl,acid} are, in many respects, similar to those of I_{Cl,acid} in cultured cells (1, 3, 15). The present study further shows that I_{Cl,acid}, unlike I_{Cl,vol} and I_{Cl,CFTR}, activates independently of the presence of intracellular ATP and that GTP-binding protein is not involved in its activation. In addition, we obtained data that indicate that hypotonic or hypertonic conditions, respectively, are inhibitory or facilitatory for the development of I_{Cl,acid}. On the other hand, I_{Cl,CFTR} induced by β-adrenergic stimulation was inhibited by acidification in a pH-dependent manner. Because an acidic environment can develop in local myocardium under pathological conditions such as myocardial ischemia, I_{Cl,acid} would play a role in regulation of cardiac electrical activity and cell volume under these pathological conditions. Preliminary accounts of this work have appeared in abstract form (26, 27).

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MATERIALS AND METHODS

Cell preparation. The Committee of Animal Welfare in Saga Medical School approved the use and treatment of all animals used in the experiments described here. The investigation conforms also with the Guiding Principles of the Physiological Society of Japan. Single cardiac myocytes from mouse (18–25 g, C57BL/6J/black inbred, male and female) and guinea pig (250–350 g, male and female) hearts were isolated with an enzymatic dispersion technique as described previously (25, 28, 29). The animals were anesthetized with pentobarbital sodium (50 mg/kg ip). The chest was opened, and the heart was rapidly removed and perfused at 37°C, using a modified Langendorff technique with a fully oxygenated physiological saline solution (PSS; see Solutions and drugs) for 2–5 min, then with a normally Ca\textsuperscript{2+}-free PSS until the heart ceased to beat, and finally with a Ca\textsuperscript{2+}-free solution containing 0.1% collagenase (CLS II, Worthington, Lakewood, NJ) and 1.0% bovine serum albumin for 10–20 min. The collagenase was then washed out of the heart with a high-K\textsuperscript{+}, low-CI\textsuperscript{-} storage solution (see Solutions and drugs). The digested atria and ventricles were separately dissected to disperse the cells in the high-K\textsuperscript{+}, low-CI\textsuperscript{-} solution. Isolated myocytes were stored in this medium at 4°C until use. Only rod-shaped atrial and ventricular myocytes with clear cross-striations and no blebs were used for experiments.

Electrophysiological techniques. The tight-seal whole cell patch-clamp technique was used to record membrane currents in isolated cells. Patch pipettes (borosilicate glass electrodes) had a tip resistance of 1–3 M\Omega when filled with pipette solution. Voltage-clamp recordings were performed with a patch-clamp amplifier (TM-1000; Actiware, Me, Tokyo, Japan), and membrane currents were filtered at 2.5 kHz and sampled at 5 kHz with an analog-to-digital converter (Digidata 1322A and pCLAMP 9.0 software (Axon Instruments, Foster City, CA). Action potentials (APs) were recorded in current-clamp configuration with a patch-clamp amplifier (CEZ-2300; Nihon Kohden, Tokyo, Japan). Depolarizing pulses of 2-ms duration, sufficient to trigger an AP, were injected at a frequency of 5 Hz. A 3 M KCl-agar bridge was set between the bath and the Ag-AgCl reference electrode to minimize changes in liquid junction potential. Unless otherwise stated, current recordings were made by applying voltage pulses of 400-ms duration to various potentials (between −100 and +120 mV in 20-mV steps) from a holding potential of 0 mV every 2 s. When necessary, current density (current magnitude per membrane capacitive area) was calculated, cell membrane capacitance being measured with pCLAMP 9.0 software. All voltage-clamp recordings were performed at room temperature and AP recordings at 36 ± 1°C.

Solutions and drugs. PSS for cell preparation contained (mM) 126 NaCl, 10 glucose, 4.4 KCl, 5.0 MgCl\textsubscript{2}, 1.5 CaCl\textsubscript{2}, 20 tauroine, 5.0 creatine, 5.0 sodium pyruvate, 1.0 NaH\textsubscript{2}PO\textsubscript{4}, and 10 HEPES, pH 7.4 adjusted with NaOH. The high-K\textsuperscript{+}, low-CI\textsuperscript{-} solution for cell storage contained (mM) 70 potassium glutamate, 20 KCl, 1.0 MgCl\textsubscript{2}, 10 KH\textsubscript{2}PO\textsubscript{4}, 10 taurine, 10 EGTA, 10 glucose, 0.1% albumin, 10 β-hydroxybutyric acid, and 10 HEPES, pH 7.2 with KOH, 320 mosM with mannitol. All bath and pipette solutions were prepared to minimize cation currents and Ca\textsuperscript{2+}-dependent currents. For mouse IC\textsubscript{L},acid and IC\textsubscript{L,CFTR} recordings, the standard bath solution contained (mM) 127 NaCl, 0.8 MgCl\textsubscript{2}, 1.0 CaCl\textsubscript{2}, 5.0 CsCl, 2.0 BaCl\textsubscript{2}, 0.2 CdCl\textsubscript{2}, 5.5 glucose, 10 HEPES, 5.0 MgATP, 0.1 Tris-GTP, and 0.01 Tris-methylene, total extracellular Cl\textsuperscript{-} concentration ([Cl\textsuperscript{-}]\textsubscript{o}) = 140 mM and pH 4.5–8.5 adjusted with t-aspartic acid or N-methyl-t-glucamine (NMDG), where appropriate. For guinea pig IC\textsubscript{L,acid} and IC\textsubscript{L,CFTR} Recordings, NaCl was totally replaced with NMDG-CI\textsuperscript{-} to inhibit an acidification-induced cationic current (17). CdCl\textsubscript{2} in these solutions was expected to inhibit IC\textsubscript{L,ir}, if any (13, 14). Low-[Cl\textsuperscript{-}] bath solutions were prepared by replacing NaCl in the standard bath solution with Na salt of various anions (aspartate−, I−, Br−, F−, and SCN−) on an equimolar basis. Hypotonic and isotonic bath solutions for IC\textsubscript{L,vol} recording were prepared by reducing NaCl concentration in the standard bath solution to 77 mOsm, with [Cl\textsuperscript{-}]\textsubscript{o} = 90 mM and 200 and 320 mosM with mannitol, respectively. Thus both hypotonic and isotonic solutions had a constant ionic strength.

The standard pipette solution contained (mM) 140 NMDG, 140 HCl, 5.0 MgATP, 5.0 EGTA, and 10 HEPES, pH 7.3 adjusted with NMDG and total intracellular Cl\textsuperscript{-} concentration ([Cl\textsuperscript{-}]\textsubscript{i}) = 140 mM. In some experiments, guanosine 5′-O-(2-thiophosphosphate) (GDP\textsubscript{S}; 1.0 mM) was added to this solution or EGTA was replaced with BAPTA on an equimolar basis. The pipette solution with 30 mM [Cl\textsuperscript{-}]\textsubscript{i} was prepared by replacing 110 mM NMDG-CI\textsuperscript{-} in the above solution with an equimolar amount of NMDG-aspartate. Osmolarity of standard bath and pipette solutions was adjusted to 320 and 290 mosM with mannitol, respectively, for inhibition of swelling-induced currents under control conditions. The pipette solution for IC\textsubscript{L,vol} recordings was prepared by reducing both NMDG and HCl in the standard pipette solution to 90 mM, pH 7.3 with NMDG and 290 mosM with mannitol. When simultaneous recordings of IC\textsubscript{L,acid} and IC\textsubscript{L,vol} were attempted (see Figs. 5–8), the bath and pipette solutions for IC\textsubscript{L,vol} recordings were used. For AP recordings, the bath solutions contained (mM) 140 NaCl, 5.4 KCl, 0.5 MgCl\textsubscript{2}, 1.8 CaCl\textsubscript{2}, 0.33 NaH\textsubscript{2}PO\textsubscript{4}, 5.5 glucose, and 5 HEPES, total [Cl\textsuperscript{-}]\textsubscript{o} = 150 mM, pH 7.4 with NaOH, and 320 mosM with mannitol. The pipette solution for AP recordings contained (mM) 110 potassium glutamate, 30 KCl, 10 NaCl, 5.0 MgATP, 0.1 Tris-GTP, 5.0 EGTA, and 10 HEPES, total [Cl\textsuperscript{-}]\textsubscript{i} = 40 mM, pH 7.3 with KOH, and 290 mosM with mannitol. Osmolarity was measured with a freezing point depression osmometer (model OM-801; Vogel, Giessen, Germany). Drugs used were glibenclamide (Sigma), DIDS (Sigma), tamoxifen (Sigma), niflumic acid (Sigma), forskolin (Sigma), 8-bromoadenosine 5′-O-(2-thiodiphosphate) (8-BrcAMP; Sigma), and isoprenaline (Daichi, Tokyo, Japan).

Data analysis. The linear fitting of the reversal potential-log [Cl\textsuperscript{-}]\textsubscript{o} relationships (see Fig. 2A) was made according to a regression analysis with the least-squares method. Data are expressed as means ± SE; n indicates the number of cells. Statistical comparisons were performed either by one-way or two-way ANOVA with post hoc test (Scheffé’s multiple comparison test) for group data as appropriate or by Student’s paired t-test when only paired groups were compared. A two-tailed probability of <0.05 was taken to indicate statistical significance.

RESULTS

Figure 1 shows the activation of whole cell currents by acidic pH\textsubscript{o} observed in a mouse ventricular cell. When pH\textsubscript{o} was changed from normal (7.4) to 6.5, the whole cell currents at both positive and negative voltages gradually increased and reached an almost steady level in ~5 min (Fig. 1A). The whole cell currents further increased on subsequent pH change to 4.5 and decreased to control level quickly (within 1 min) on resumption of pH\textsubscript{o} 7.4. The I-V relationships of the acidic pH\textsubscript{o}-activated current exhibited a clear outward rectification (Fig. 1, Bc and C). During the applied voltage pulses, the current was largely time independent, but a time-dependent slow activation was noted during large positive pulses (e.g., +100 and +120 mV; Fig. 1Bc). Using solutions with various pH\textsubscript{o}, we obtained pH\textsubscript{o}-response relationships for acidic pH\textsubscript{o}-induced (difference) currents at +120, +40, and ~80 mV (Fig. 1D). At all voltage steps, the threshold pH value for current activation appeared to be ~pH 7, and the maximum activation was obtained at ~pH 4.5. An analysis with the Hill equation for the currents at +120 mV revealed the pH value for half-maximal activation (EC\textsubscript{50}) and Hill coefficient (n\textsubscript{H}) to be pH 5.89 and 1.12, respectively. Such acidic pH\textsubscript{o}-induced currents were observed in 24 of 26 ventricular cells examined (9 mice). Similar currents were also observed (data not shown) in...
Although pipette solutions with lower pH values were not examined, the above findings might suggest that the protonation site is located outside the membrane. We next compared the effects of several Cl⁻ channel inhibitors (DIDS, glibenclamide, niflumic acid, and tamoxifen) on I_{Cl,acid}. To activate I_{Cl,acid} in these experiments, we used a bath solution with pH 5.5, because high (50–100 μM) concentra-

Fig. 1. Activation of whole cell currents by acidic extracellular pH (pHₐ) in mouse ventricular myocytes. A: time course of activation of whole cell currents recorded in a cell under symmetrical Cl⁻ concentration ([Cl⁻]o: 140 mM) conditions during application of acidic bathing solution. In this and subsequent similar figures, currents were measured at +80 (a) and −80 (c) mV, with the pulse protocol shown in the inset. pH of the bathing solution was changed from 7.4 to 6.5 or 4.5 during the time indicated by bar. B: records of membrane currents obtained at the time points indicated by a, b, and c in A. C: mean steady-state current-voltage (I-V) relationships (n = 24) of whole cell currents obtained at pH 7.4 and pH 4.5. ‘‘*’’Significant change at P < 0.05 and 0.01, respectively. D: pHₐ-response relationship of acidic pHₐ-induced (difference) currents (∆I). Each point represents mean of data obtained in 4 different cells, and curves were obtained by fitting the data points to the Hill equation. Data were obtained at +120, +40, and −80 mV at steady state.

mouse atrial cells (n = 6) and in atrial (n = 4) and ventricular (n = 10) cells from guinea pig. We could not detect any outwardly rectifying whole cell current in cells dialyzed with an acidic (pH 6.5) pipette solution and perfused at pH 7.4, but exposure of these cells to an acidic (pH 6.5) pipette solution and perfused at pH 7.4, but exposure of these cells to an acidic (pH 6.5) pipette solution and perfused at pH 4.5, +80 mV, with the pulse protocol shown in the inset. pH of the bathing solution was changed from 7.4 to 6.5 or 4.5 during the time indicated by bar. B: records of membrane currents obtained at the time points indicated by a, b, and c in A. C: mean steady-state current-voltage (I-V) relationships (n = 24) of whole cell currents obtained at pH 7.4 and pH 4.5. ‘‘*’’Significant change at P < 0.05 and 0.01, respectively. D: pHₐ-response relationship of acidic pHₐ-induced (difference) currents (∆I). Each point represents mean of data obtained in 4 different cells, and curves were obtained by fitting the data points to the Hill equation. Data were obtained at +120, +40, and −80 mV at steady state.

The reversal potential (V_{rev}) of the acidic pHₐ-induced current depended on [Cl⁻]o. Figure 2A shows V_{rev}-log [Cl⁻]o relationships obtained at three different [Cl⁻]o, with a pipette (internal) solution containing 30 mM Cl⁻. The regression line had a slope of 58.1 mV per 10-fold change in [Cl⁻]o, indicating that chloride ions were the main charge carrier of this current. In the preceding experiments (Fig. 1C), V_{rev} was 3.1 ± 1.9 mV (n = 24), and this value is also close to the Cl⁻ equilibrium potential (E_{Cl} = 0 mV) predicted for those symmetrical [Cl⁻] conditions. Thus we designated the acidic pHₐ-induced current as I_{Cl,acid}. To examine the anion selectivity of I_{Cl,acid}, we measured I-V relationships of I_{Cl,acid} in low-[Cl⁻]o solutions containing high concentrations of different anions. Examples are shown in Fig. 2B, in which I-V relationships obtained in Cl⁻, I⁻, and aspartate-rich solutions are illustrated. Quantitative analysis with the Goldman-Hodgkin-Katz equation revealed that the relative permeability (P) for each anion X⁻ to Cl⁻ (Pₓ/P_{Cl}) was SCN⁻ > Br⁻ > I⁻ > Cl⁻ > F⁻ > aspartate (Fig. 2C).

We next compared the effects of several Cl⁻ channel inhibitors (DIDS, glibenclamide, niflumic acid, and tamoxifen) on I_{Cl,acid}. To activate I_{Cl,acid} in these experiments, we used a bath solution with pH 5.5, because high (50–100 μM) concentra-

Fig. 2. Anion sensitivity of acidic pHₐ-induced currents. A: reversal potential (V_{rev})-log extracellular [Cl⁻]o relationship of acidic pHₐ-induced currents (difference currents) obtained with 30 mM intracellular [Cl⁻]o ([Cl⁻]i). Data were obtained in 4–6 cells exposed to pH 4.5 at each [Cl⁻]o. Linear regression analysis revealed V_{rev} = 58.1 log (34.7/[Cl⁻]o). B: mean I-V relationships of acidic pHₐ-induced (difference) current obtained in solutions containing different anion species at pH 4.5. I⁻-rich (I⁻) or aspartate-rich (Asp⁻) solution was made by replacing 127 of 140 mM NaCl in normal-Cl⁻ solution ([Cl⁻]o) with an equimolar amount of NaI or Na-aspartate, respectively; n = 4 for each solution. C: anion permeability (P) sequence of the acidic pHₐ-induced current. Experiments similar to those shown in B were performed with various anion species (anion X⁻) as indicated, and V_{rev} was determined from the I-V relation of the difference current for each X⁻-rich solution. The relative P for each anion X⁻ to Cl⁻ (Pₓ/P_{Cl}) was calculated with the Goldman-Hodgkin-Katz equation. Number of cells examined in each solution is given in parentheses.
tions of glibenclamide were insoluble at pH 4.5. DIDS, glibenclamide, and niflumic acid were soluble at a concentration of 100 μM and tamoxifen at 10 μM. Figure 3A shows an example of such experiments. One hundred micromolar DIDS strongly inhibited the outward \( I_{\text{Cl,acid}} \), whereas it inhibited the inward \( I_{\text{Cl,Ca}} \) only weakly, indicating that DIDS exerted a voltage-independent inhibition on \( I_{\text{Cl,acid}} \). Figure 3B summarizes the effect of the inhibitors on \( I_{\text{Cl,acid}} \). Glibenclamide and niflumic acid moderately inhibited \( I_{\text{Cl,acid}} \) in a voltage-independent manner, whereas \( I_{\text{Cl,acid}} \) was little sensitive to tamoxifen. On the other hand, DIDS (100 μM) and tamoxifen (10 μM) exerted little effect on the background current at pH 7.4 (data not shown), suggesting that any inhibitor-sensitive current like \( I_{\text{Cl,vol}} \) was absent at least at pH 7.4.

The β-adrenergic stimulation of \( I_{\text{Cl,CFTR}} \) involves activation of the receptor-coupled GTP-binding protein (G protein), whereas several cardiac membrane currents such as \( I_{\text{Cl,Ca}} \) depend on intracellular \( Ca^{2+} \). We examined how the tonicity of the bathing solution influenced \( I_{\text{Cl,acid}} \). \( I_{\text{Cl,acid}} \) could also be activated in cells dialyzed with a pipette solution in which the intracellular \( Ca^{2+} \) concentration ([Ca^{2+}]) was 100 μM and tamoxifen (10 μM) exerted little effect on the background current at pH 7.4 (data not shown), suggesting that any inhibitor-sensitive current like \( I_{\text{Cl,vol}} \) was absent at least at pH 7.4.

The activation of \( I_{\text{Cl,CFTR}} \) (18) and \( I_{\text{Cl,vol}} \) (23) requires the presence of intracellular ATP. The suppression of the activation of these two currents in ATP-depleted cells was confirmed also in the present study (data not shown). In contrast, \( I_{\text{Cl,acid}} \) could be recorded in the absence of intracellular ATP (see Figs. 5 and 6). Similar results were obtained in 5 mM adenosine 5’-(β,γ-imido)triphosphate (a nonhydrolyzable ATP analog)-loaded cells (data not shown). These may indicate that ATP hydrolysis, ATP-mediated phosphorylation, or related reactions are not involved in the activation process of \( I_{\text{Cl,acid}} \).

The biophysical and pharmacological properties of \( I_{\text{Cl,acid}} \) noted above did not appear to be the same as those of other cardiac \( Cl^- \) currents such as \( I_{\text{Cl,vol}} \) and \( I_{\text{Cl,CFTR}} \). In the following experiments, we attempted to further characterize \( I_{\text{Cl,acid}} \) compared with the properties of \( I_{\text{Cl,vol}} \) and \( I_{\text{Cl,CFTR}} \). First, we examined how the tonicity of the bathing solution influenced the activation of \( I_{\text{Cl,acid}} \) in ATP-depleted cells. In these experiments, current measurements were begun at least 10 min after establishment of the whole cell configuration with ATP-free pipette solutions. In the experiment shown in Fig. 5A, the cell was first exposed to acidic (pH 4.5) isotonic (320 mosM) solution, which resulted in a development of \( I_{\text{Cl,acid}} \) (data not shown). In contrast, the size of \( I_{\text{Cl,acid}} \) was similar at both these two \([Ca^{2+}]_i\) (data not shown), and the size of \( I_{\text{Cl,acid}} \) was similar at both these two \([Ca^{2+}]_i\), suggesting that \( I_{\text{Cl,acid}} \) activation is independent of intracellular \( Ca^{2+} \).

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Tamoxifen is a well-known inhibitor of $I_{\text{Cl,vol}}$. We confirmed that this drug greatly attenuated hypotonicity-induced $I_{\text{Cl,vol}}$ (4 cells), 10 μM tamoxifen reducing $I_{\text{Cl,vol}}$ by >60% (data not shown). Curiously, this drug was found to increase $I_{\text{Cl,acid}}$ in ATP-depleted cells under hypotonic conditions. As shown in Fig. 6A, when tamoxifen was applied to the ATP-depleted cell in acidic hypotonic solution, there was an increase in the whole cell currents (Fig. 6, Ab and Ac). The “tamoxifen-induced” current showed a time-dependent activation at positive potentials (Fig. 6Ad), and its $I-V$ relation exhibited outward rectification (Fig. 6B), suggesting that this current represented a fraction of $I_{\text{Cl,acid}}$. The mechanism underlying this response is unclear. The hypotonicity-induced cell swelling might be related to this tamoxifen action, but we did not further investigate this point.

We next examined how $I_{\text{Cl,acid}}$ and $I_{\text{Cl,vol}}$ developed simultaneously, using cells dialyzed with ATP-containing pipette solution. In the experiment shown in Fig. 7A the cell was first exposed to hypotonic solution, and this resulted in development of the $I_{\text{Cl,vol}}$, in which a time-dependent inactivation is barely visible at positive potentials (Fig. 7Bb). The $I-V$ relationship of $I_{\text{Cl,vol}}$ is shown in Fig. 7D. When pH of the hypotonic solution was changed from 7.4 to 4.5, there was a sizable increase in the whole cell currents (Fig. 7, A and Bc). It is noteworthy that the increased outward currents, like $I_{\text{Cl,acid}}$ shown above (e.g., Fig. 1Bc), exhibited a time-dependent activation at large positive potentials (Fig. 7Bc). The acid-induced current (difference current) obtained during the hypotonic challenge and its $I-V$ relationships are shown in Fig. 7, Bb and E, respectively.
absence of tamoxifen. E: mean I-V relationship of the hypotonic solution was 320 mosM, and that of the hypotonic solution was 200 mosM.

conditions. Hypotonic solution and acidic hypotonic (pH 4.5) solution were subjected to that shown in Fig. 7D). However, this agent did not inhibit the development of acid-induced current (Fig. 7C). The acid-induced current showed a time-dependent activation at large positive potentials (Fig. 7Cd), and its magnitude was comparable to that seen without tamoxifen (Fig. 7E).

Lambert and Oberwinkler (15) observed that when hypotonic solution was switched to acidic hypotonic solution, there was a decrease in the outward I_{Cl,vol} before development of I_{Cl,acid}. We did not consistently observe such a phenomenon in the present study, and the effect of acidification on I_{Cl,vol} in our preparation is unclear. Hence the difference current shown in Fig. 7E cannot be regarded as representing the true magnitude of I_{Cl,acid}. However, the density of the acid-induced currents (Fig. 7E) is comparable to that of I_{Cl,acid} obtained in ATP-depleted cells (Fig. 7F) in which I_{Cl,vol} was presumed to be absent. We consider that the acid-induced currents obtained here (Fig. 7, Bd and E) represent largely, if not entirely, I_{Cl,acid} and that acidification exerts no profound effect on I_{Cl,vol}.

In the above experiments, I_{Cl,acid} developed in the presence of I_{Cl,vol}. We also observed that I_{Cl,vol} developed in the presence of I_{Cl,acid}. In the experiment shown in Fig. 8, the cell was first exposed to acidic solution to activate I_{Cl,acid} (Fig. 8Bb). When an acidic hypotonic solution was consecutively introduced, the whole cell currents initially decreased (Fig. 8, A and Bc) and increased thereafter, the increase overwhelming the current level caused by acidification alone (Fig. 8, A and Bd). It is conceivable that I_{Cl,vol} developed in addition to I_{Cl,acid} at this situation. On the other hand, the current decrease observed shortly after acidic hypotonic solution (Fig. 8Bc) was considered to reflect the inhibitory effect of hypotonic condition on I_{Cl,acid}, which was noted above (Fig. 5). The I-V relationship of the whole cell currents obtained during the course of the experiment is shown in Fig. 8C and that of hypotonicity-induced currents obtained at acidic pH in Fig. 8D. We conclude that I_{Cl,acid} and I_{Cl,vol} can develop in an additive manner in cardiac cells.

We next examined the effects of acidification on I_{Cl,CFTR}. For this purpose, we used guinea pig ventricular cells, because these cells, unlike mouse cells (5, 16), develop I_{Cl,CFTR} in response to β-adrenergic stimulation (9, 11, 12). In the experiment shown in Fig. 9A, application of 1 μM isoproterenol to the cells induced I_{Cl,CFTR} (Fig. 9Ab). Subsequent acidification in the presence of isoproterenol clearly increased the outward currents, and the increased currents showed time-dependent activation at large positive potentials, but the inward currents increased only slightly in this situation (Fig. 9Ac), the whole cell current revealing clear outward rectification. Curiously, withdrawal of isoproterenol from the acidic solution had little effect on the currents at any voltage (Fig. 9, Ac and d). It appears that acidification to pH 4.5 eliminated I_{Cl,CFTR}, while inducing I_{Cl,acid}.

The effect of acidic pH on I_{Cl,CFTR} was further studied at pH 6.5, 5.5, and 4.5 in the presence of 100 μM DIDS, the latter being expected to suppress I_{Cl,acid}. In the presence of DIDS, the lower the pH value of agonist-containing solution the smaller the whole cell current (Fig. 9B), confirming the acid-induced inhibition of I_{Cl,CFTR}. The I-V relationship of the current component suppressed by acidification to pH 4.5 (difference current) is shown in Fig. 9C. V_rev of this component was near current (obtained in these experiments) are shown in Fig. 7, C, D, and E, respectively. Tamoxifen inhibited the development of I_{Cl,vol} as expected (Fig. 7D). However, this agent did not inhibit the development of acid-induced current (Fig. 7C).

Similar experiments were also performed in the continuous presence of tamoxifen (10 μM) with the experimental protocol shown in Fig. 7A. The records of membrane currents, I-V relationships of I_{Cl,vol}, and acid-induced current (difference current) obtained in these experiments are shown in Fig. 7, C, D, and E, respectively. Tamoxifen inhibited the development of I_{Cl,vol} as expected (Fig. 7D). However, this agent did not inhibit the development of acid-induced current (Fig. 7C).

Fig. 7. Activation of I_{Cl,acid} in the presence of volume-regulated Cl− current (I_{Cl,vol}) in cells with 5 mM [ATP]. Cells were dialyzed with pipette solution containing 5 mM ATP. A: time course of activation of whole cell currents recorded during hypotonic challenge followed by acidification. Currents were measured at 0.8 and 0.2 mV in a cell under symmetrical [Cl−] (90 mM) conditions. Hypotonic solution and acidic hypotonic (pH 4.5) solution were applied during the time indicated by bars. The osmolality of the isotonic solution was 320 mosM, and that of the hypotonic solution was 200 mosM. B: records of membrane currents obtained at the time points indicated by a, b, and c and d. Hypotonic challenge induced I_{Cl,vol} (b) and the additional acidification further increased the whole cell currents (c). The difference current (c − b) is shown in d. C: records of membrane currents obtained in an experiment similar to that shown in A. The experimental protocol was the same as in A, but here tamoxifen (10 μM) was present throughout. D: mean I-V relationships (n = 4) of I_{Cl,vol} (difference current, b − a in B and C) obtained in the presence or absence of tamoxifen. E: mean I-V relationships (n = 4) of acid-induced currents obtained in the presence of I_{Cl,vol} (d in B and C) with and without tamoxifen.
0 mV, and the outward portion of I-V relation was almost linear, in line with the property of $I_{\text{Cl,CFTR}}$. The inward portion exhibited a rectification-like bending. This bending was attributed to an insufficient inhibition of $I_{\text{Cl,acid}}$ by DIDS at negative voltages (Fig. 3). The inward currents at pH$_{o}$ 4.5 (Fig. 9Bd) may have contained a substantial amount of $I_{\text{Cl,acid}}$, which would lead to a decrease of the difference currents because the current at pH$_{o}$ 4.5 was subtracted from that at pH$_{o}$ 7.4. A similar inhibitory effect of acidic pH$_{o}$ on $I_{\text{CL,CFTR}}$ was observed when $I_{\text{CL,CFTR}}$ was activated by application of other PKA activators, 5.5 mM forskolin and 0.5 mM 8-Br-cAMP (data not shown).

Figure 9D shows the relationship between density of the outward current at $+100$ mV and the pH$_{o}$ value. If we consider that the outward whole cell current observed at a given pH in the presence of both isoprenaline and DIDS largely represents $I_{\text{Cl,CFTR}}$, the data shown in Fig. 9D can be a measure of the pH$_{o}$-response relationship for $I_{\text{Cl,CFTR}}$. We conclude that $I_{\text{Cl,acid}}$ is distinct from $I_{\text{Cl,CFTR}}$ and that acidic pH$_{o}$ depresses the latter, in agreement with earlier results (17). In additional experiments in which mouse ventricular cells were used, application of isoprenaline (1 μM), which did not induce $I_{\text{Cl,CFTR}}$ in these cells, had no effect on $I_{\text{Cl,acid}}$ ($n = 6$, data not shown), suggesting that $I_{\text{Cl,acid}}$ is independent of the β-adrenoceptor-PKA system.
DISCUSSION

$I_{\text{Cl,acid}}$ observed in the present study showed the following properties. The $I-V$ relation of $I_{\text{Cl,acid}}$ outwardly rectifies under symmetrical $[\text{Cl}^-]$ conditions (Fig. 1C). $I_{\text{Cl,acid}}$ shows a time-dependent activation at large positive potentials (Fig. 1B). It is sensitive to DIDS, glibenclamide, and niflumic acid but insensitive to tamoxifen under isotonic conditions (Fig. 3B) and is independent of intracellular calcium ions, ATP, and G protein (Figs. 4 and 5). The relative permeability sequence for anions appears to be $\text{SCN}^- > \text{Br}^- > \Gamma^- > \text{Cl}^- > \text{F}^- > \text{aspartate}$ (Fig. 2C). The magnitude of $I_{\text{Cl,acid}}$ appears to depend on osmotic conditions in ATP-depleted cells (Fig. 5). Tamoxifen increases $I_{\text{Cl,acid}}$ under hypotonic conditions (Fig. 6), in contrast with its effect on $I_{\text{Cl,acid}}$ under isotonic conditions. These properties of $I_{\text{Cl,acid}}$ are totally or partially different from those of other cardiac Cl$^-$ currents ($I_{\text{Cl,CFTR}}, I_{\text{Cl,vol}}, I_{\text{Cl,Ca}},$ and $I_{\text{Cl,ir}}$) identified so far. $I_{\text{Cl,ir}}$ (4, 13, 14) increases at low pH$_{\text{r}}$ and on cell swelling, but it inwardly rectifies. $I_{\text{Cl,Ca}}$ (9, 11, 24) requires an elevation of $[\text{Ca}^{2+}]$, for its activation. $I_{\text{Cl,vol}}$ (8, 9, 11, 29) exhibits a time-dependent inactivation at positive potentials and is sensitive to both DIDS and tamoxifen. $I_{\text{Cl,CFTR}}$ (9, 11) shows little rectification under symmetrical $[\text{Cl}^-]$ conditions and is insensitive to DIDS. With respect to intracellular regulation, activation of $I_{\text{Cl,CFTR}}$ (18) and $I_{\text{Cl,vol}}$ (23) has been shown to depend on intracellular ATP. Furthermore, $I_{\text{Cl,acid}}$ appears to develop independently of $I_{\text{Cl,vol}}$ (Figs. 7 and 8) or $I_{\text{Cl,CFTR}}$ (Fig. 9).

Some technical considerations must be made. We determined the pH dependence and $V_{\text{rev}}$ of $I_{\text{Cl,acid}}$ by using acid-induced currents (difference currents), and it was assumed that the background currents other than $I_{\text{Cl,acid}}$ were insensitive to acidification. In reality, however, the magnitude of the background currents might change depending on acidity. For example, although DIDS and tamoxifen were ineffective on the background currents at neutral pH, it cannot be ruled out that some inhibitor-sensitive or -insensitive background currents developed at acidic pH. Therefore, our data on the pH dependence and permeability sequence of $I_{\text{Cl,acid}}$ might contain some inaccuracies, although, as pointed out by Lambert and Oberwinkler (15), the large currents of $I_{\text{Cl,acid}}$ should minimally be affected by such changes in background currents.

$I_{\text{Cl,acid}}$ has been recorded in many types of cultured cells derived from nonexcitable cells (1, 3, 15, 19). The current in these cells has common features. It exhibits outward rectification in its $I-V$ relation, time-dependent activation during depolarization, and independence of activation on intracellular $[\text{Ca}^{2+}]$. The sensitivity of $I_{\text{Cl,acid}}$ to DIDS (1, 15, 19), niflumic acid (19), and glibenclamide (2) has been demonstrated in some cells. The capability of $I_{\text{Cl,acid}}$ channels to carry several kinds of anions has also been noted (1, 15, 19). These properties are in agreement with those of cardiac $I_{\text{Cl,acid}}$ observed in the present study. As with anion selectivity, $\Gamma^-$ and $\text{Br}^-$ appear to be more permeable than Cl$^-$ in cardiac $I_{\text{Cl,acid}}$. Although this feature resembles that for $I_{\text{Cl,acid}}$ in HEK293 cells (15, 19), a reverse relationship ($\Gamma^- > \text{Br}^- > \Gamma^-$) has been reported for $I_{\text{Cl,acid}}$ in Sertoli cells (1). The nature of this difference is unknown (see Ref. 15).

Nobles et al. (19) observed a tamoxifen-resistant $I_{\text{Cl,acid}}$ in cultured cells. However, they considered that this $I_{\text{Cl,acid}}$ was a manifestation of $I_{\text{Cl,vol}}$ channels. In their view, the biophysical as well as pharmacological properties of $I_{\text{Cl,vol}}$ channels are altered by acidic pH$_{\text{r}}$, so that the channels can be activated by acidic pH$_{\text{r}}$ under isotonic conditions. This proposal might not be incompatible with the feature of $I_{\text{Cl,acid}}$ mentioned above. Recently, however, Lambert and Oberwinkler (15) extensively examined $I_{\text{Cl,acid}}$ and $I_{\text{Cl,vol}}$ in HEK293 cells. On the basis of their detailed analysis including examinations of the single-channel events, the effects of acidification on $I_{\text{Cl,vol}}$, and the effects of train of depolarizing pulses on $I_{\text{Cl,vol}}$ at acidic pH$_{\text{r}}$ (see introduction), they concluded that $I_{\text{Cl,acid}}$ and $I_{\text{Cl,vol}}$ are derived from different channels.

In our study, $I_{\text{Cl,acid}}$ and $I_{\text{Cl,vol}}$ appeared to develop in an additive fashion in acidic hypotonic solutions, with each current preserving its own voltage-dependent behavior, $I_{\text{Cl,acid}}$ showing time-dependent activation and $I_{\text{Cl,vol}}$ time-dependent inactivation at positive voltages (Figs. 7 and 8). We observed...
an inhibitory effect of hypotonic condition on $I_{\text{Cl,acid}}$ (Figs. 5 and 8). The mechanism underlying this effect is unclear. Nevertheless, as discussed by Lambert and Oberwinkler (15), it seems difficult to explain the biphasic change of the whole cell current observed during acidic hypotonic challenge (Fig. 8) by simply assuming that $I_{\text{Cl,acid}}$ channels are acidification-modified $I_{\text{Cl,vol}}$ channels. In addition, $I_{\text{Cl,acid}}$, unlike $I_{\text{Cl,vol}}$, can develop independent of intracellular ATP (Figs. 5 and 6), suggesting involvement of different intracellular regulatory mechanisms in the activation of these two currents. The simplest explanation of our findings may be that $I_{\text{Cl,acid}}$ can develop independently of $I_{\text{Cl,vol}}$, in agreement with the conclusion of Lambert and Oberwinkler (15).

The mechanism of activation of $I_{\text{Cl,acid}}$ by acidic pH is unclear. Our study suggested that popular intracellular signaling molecules such as G protein, PKA, and ATP were not required for activation of $I_{\text{Cl,acid}}$. The pH-response relationship for $I_{\text{Cl,acid}}$ obtained in our study revealed that $E_{\text{Cl,50}}$ is $\sim$ pH 5.9 with a threshold pH value of $\sim$ pH 7 and $n_H$ of $\sim$1.1 (Fig. 1D).

The feature is somewhat different in HEK293 cells, in which $E_{\text{Cl,50}}$ of pH 5.1, threshold of $\sim$ pH 5.5, and $n_H$ of 3.6 have been reported (15). The difference might indicate a difference in the molecular structure of the $I_{\text{Cl,acid}}$ channel itself or related regulatory proteins. Further studies are necessary to elucidate this point.

The activation of cardiac $I_{\text{Cl,acid}}$ at severely acidic pH with a threshold of $\sim$ pH 7 and $E_{\text{Cl,50}}$ of pH 5.9 means that the $I_{\text{Cl,acid}}$ channels can hardly function under physiological conditions. However, local external acidosis is frequently induced by myocardial ischemia and is known to alter properties of several ion channels and transporters (10, 13, 14, 21), which might lead to development of cardiac arrhythmias (21). Yan and Kleber (30) reported that in blood-perfused rabbit papillary muscles, no-flow ischemia for 14 min resulted in an acidification of the extracellular space up to pH 6.64–6.30, depending on $P_{\text{CO}_2}$. Essentially similar changes in pH have been observed in pig heart in vivo after 10-min occlusion of a branch with a threshold pH value of 28). Because $I_{\text{Cl,acid}}$ suggests that activation of $I_{\text{Cl,acid}}$ might play a cell volume-regulatory role in place of $I_{\text{Cl,vol}}$ during myocardial acidosis. As with the pH dependence of $I_{\text{Cl,acid}}$, it should be noted that the $I_{\text{Cl,acid}}$ channels might change their properties depending on unknown cellular signaling events so that they would become more active under less acidic conditions. Obviously, further studies are necessary to elucidate the physiological significance of $I_{\text{Cl,acid}}$, including its roles in cardiac function under pathological conditions.

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**REFERENCES**


