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Intracellular Alkalinization Enhances Inward Rectifier K^+ Current in Retinal Horizontal Cells of Catfish

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ABSTRACT—Isolated cone-driven horizontal cells dissociated from catfish retina were voltage-clamped using the whole-cell patch-clamp technique. The effects of acidification and alkalinization on an anomalous type, inwardly-rectifying K^+ current (IRK^+) were investigated. The magnitude of IRK^+ was enhanced by raising the intracellular pH above 7.4, however, in contrast, intracellular acidification had little effect on this current. The range over which intracellular pH ($[pH]_i$) modulates IRK^+ is different from that for modulation of a sustained high-voltage activated calcium current in these same cells and also for proton-sensitive, inward rectifier currents in starfish oocytes, skeletal muscle and heart myocytes.

INTRODUCTION

In the vertebrate retina horizontal cells are second-order neurons that receive glutamatergic input from photoreceptors. By feeding signals back to photoreceptors, horizontal cells play important roles in contrast enhancement and in adjusting retinal sensitivity to ambient background light [1, 24]. Several different voltage-dependent ionic conductances have been identified in horizontal cells: a TTX-sensitive sodium current, a sustained calcium current and three types of potassium currents [9, 14, 16, 18, 21]. Several of these currents are activated in the voltage ranges normally transgressed by horizontal cell membrane potential in light and dark. The inward rectifier K^+ current (IRK^+) is activated by hyperpolarization that is evoked by bright light. By analogy to a similar current identified in photoreceptors [2], activation of the inward rectifier in horizontal cells would restrict the amount of hyperpolarization induced by bright flashes or steps of light.

Recently, Takahashi, Dixon and Copenhagen [20] found that the high voltage-activated calcium current in cone-driven catfish horizontal cells is modulated by intracellular pH ($[pH]_i$). A later study revealed that this same calcium current was suppressed by micromolar concentrations of L-glutamate via an alteration in $[pH]_i$ [8]. During the course of the previous studies it was noted that $[pH]_i$ altered the magnitude of the inward rectifier current. In this present report we characterize the pH-dependency of IRK^+ and explore the possibility that extracellular glutamate, by changing $[pH]_i$, modulates this current.

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MATERIALS AND METHODS

Catfish (*Ictalurus punctatus*) were dark-adapted for several hours, cooled, swiftly decapitated with a guillotine and pithed. Eyes were enucleated and hemisected, then the retinae were removed from the pigment epithelium. Horizontal cells were dissociated using papain and mechanical trituration following the procedure of Tachibana [17]. Cells were kept in a modified L-15 media (GIBCO) at 12°C for periods ranging from 2 to 14 days. Patch-clamp recordings were done at room temperature in a superfusion chamber fitted to the stage of an inverted microscope with phase-contrast optics (Nikon, TMD).

Patch pipettes were fabricated on a Brown-Flaming type puller (Sutter Instrument Co., P-87, Novato, CA) from 1.5 mm diameter borosilicate glass (Garner glass company, Claremont, CA) and had resistances ranging from 5 to 10MΩ. Patch recordings were performed in the whole-cell voltage-clamp configuration. The standard pipette solution contained (in mM): K-gluconate 140, NaCl 4.0, $MgCl_2$ 2.0, $CaCl_2$ 2.0, EGTA 10 (pH: 7.4). Although the pipette solution contained no pH-buffer, the pH of the solution was stably maintained for at least five hours. In a high-pH buffered pipette solution, 25 mM-HEPES was added to the standard solution, and K-gluconate was reduced to 125 mM to maintain the same osmolality. For recording sustained high-voltage activated calcium currents (I_{Ca}), ATP (Na salt, 1 mM) and GTP (Na salt, 0.1 mM) were added to the standard pipette solution, and 20 mM tetraethylammonium chloride (TEA-Cl) was substituted for the same amount of an extracellular NaCl. The control saline contained (in mM): NaCl 125, KCl 2.6, $MgCl_2$ 1.0, $CaCl_2$ 2.5, Glucose 15, and HEPES 10. Solution changes were performed by manually switching a four-way stop cock (Hamilton, HV PD4-5, NV) connected to the chamber infusion line. Osmolalities of pipette and external salines were measured daily and were approximately 290 mOsm/Kg. The pH of pipette and external solutions were 7.4 and 7.8, respectively. Previous studies using pH indicator dyes revealed that these cells regulated $[pH]_i$ to 7.4 when bathed in saline close to the *in vivo* extracellular pH ($[pH]_o$) of 7.8 [19, 20]. Liquid junction potentials were measured and all potentials shown in the figures have been appropriately corrected.

Chemicals were purchased from Sigma and Aldrich.

RESULTS

IRK^+ in retinal horizontal cells is activated by hyperpolarization, is sustained and is suppressed by CsCl [16, 18]. In the present experiments, using our standard saline and pipette solutions, IRK^+ was activated at potentials hyperpolarized from approximately −90 mV. Figure 1 (control plot) shows IRK^+ in response to a voltage ramp from −134 mV to −14 mV. The top set of traces in the inset shows the current response to a series of 5 200ms voltage pulses from a holding potential of −14 mV to a range of potentials from −54 mV to −134 mV. The currents evoked in response to voltage pulses are sustained and the steady state amplitudes, shown by the X's, superimpose directly on the ramp response. Peak amplitudes of IRK^+ averaged 795±194 pA (mean±SD) at −134 mV (n=24). Both the ramp and pulse responses show that the inward current at potentials hyperpolarized beyond −90 mV is strongly suppressed in the presence of 10 mM CsCl. CsCl also appeared to block an outward current that was activated throughout the voltage range of the experiments. The characteristics of this outward current were not investigated further in this present study. Because the pulse and ramp protocols resulted in very similar current-voltage relationship, we used ramp protocols to measure IRK^+ in all subsequent experiments.

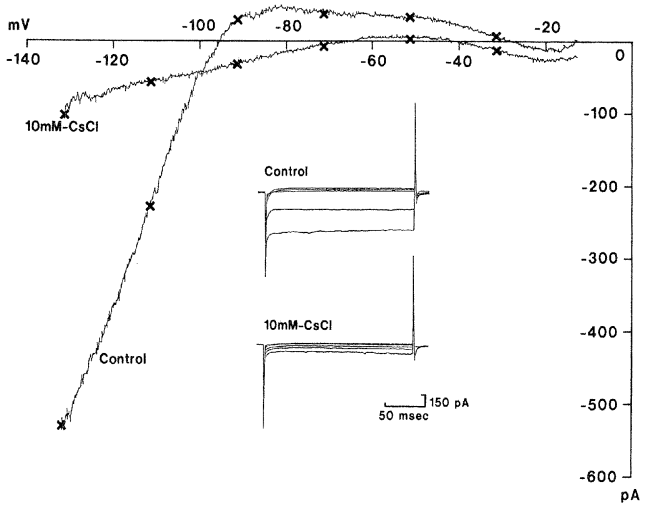

FIG. 1. Blockade of IRK^+ by 10 mM CsCl demonstrated in voltage ramps and pulses. Current-voltage curves were generated by voltage ramps from −134 mV to −14 mV (60 mV/sec). In control saline, IRK^+ was activated at about −90 mV and increased monotonically to −550 pA at −134 mV. This current was suppressed by 10 mM CsCl in the external saline. Insets. Currents to voltage pulses from −54 mV to −134 mV in 20 mV decrements from a holding voltage of −14 mV are shown. The amplitudes of currents measured with the voltage-pulses were superimposed on those measured with voltage-ramps (X's).

Effect of intracellular acidification

To study the effects of changes in $[pH]_i$ while holding $[pH]_o$ constant one can perfuse cells with weak acids, such as sodium acetate (Na-acetate), that acidifies cells, or weak bases such as ammonium chloride (NH_4Cl) that alkalinizes cells upon application, and produces a rebound acidification upon washout [4]. Previous studies using patch pipettes filled with the pH indicator dye BCECF showed that superfusion of catfish horizontal cells with 20 mM Na-acetate could acidify them by 0.3 to 0.6 pH units [20]. In the present study when horizontal cells were superfused with a saline containing 20 mM Na-acetate, no significant effect was observed on IRK^+. The change of inward current at −134 mV was 4.5 ±4.3% (n=20). Next cells were superfused with a saline containing a much higher amount of Na-acetate, in the hope of leading to the larger intracellular acidification. In 50 mM Na-acetate saline, the amplitude of IRK^+ was reduced by

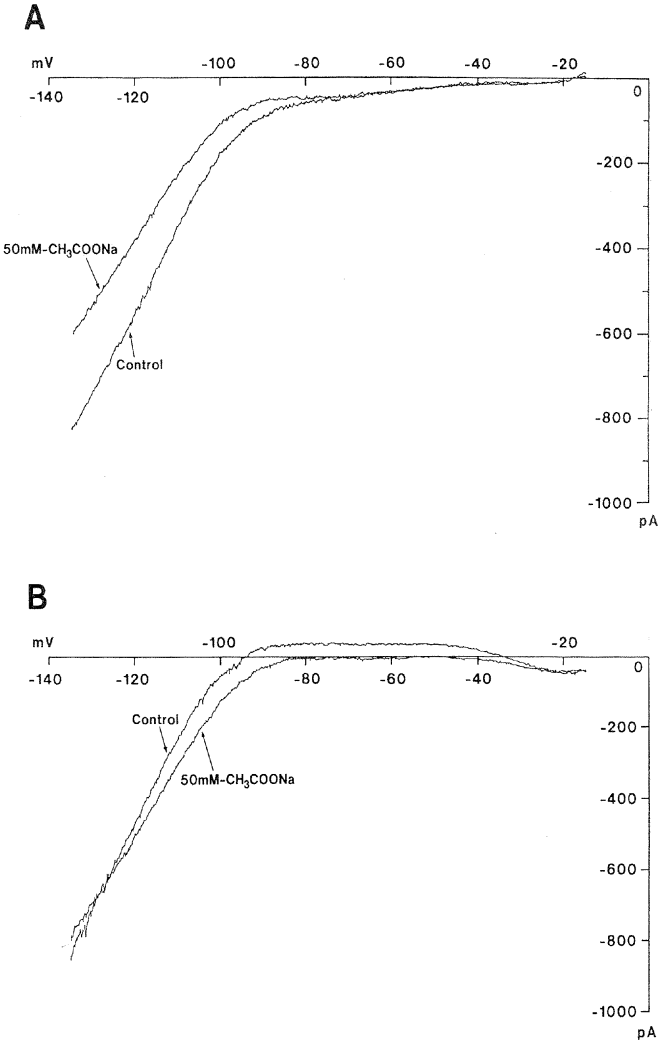

FIG. 2. Effect of 50 mM Na-acetate on IRK^+. In A, using control pipette solutions the peak current at −134 mV was reduced by about 26%. In contrast, when a pipette solution containing 25 mM HEPES was used, no significant effect of Na-acetate on IRK^+ was observed (B).

$15.1 \pm 7.4\%$ (n=17) at -134 mV. Figure 2A shows IRK^+ in control saline and in 50 mM Na-acetate saline. In this particular cell IRK^+ was reduced by 25%. However IRK^+ rarely recovered to control level following the washout of 50 mM Na-acetate. The reduction of IRK^+ in 50 mM Na-acetate was blocked when intracellular pH buffering capacity was raised; when the pipette solution contained 25 mM HEPES, 50 mM Na-acetate suppressed IRK^+ minimally ($2.4 \pm 3.4\%$, n=27) at -134 mV (Fig. 2B). These results suggest that large acidification can slightly reduce IRK^+, however the sensitivity to acidification is much less than that of I_{Ca} in the same cells [20]. We surmised that the gradual loss of IRK^+ and eventual cell death in 50 mM Na-acetate probably resulted from cellular acidification. As evidence, we found that when cells are recorded with 25 mM HEPES intracellularly, instead of HEPES-free, IRK^+ remained stable for much longer periods of time in 50 mM Na-acetate.

Figure 3 illustrates that IRK^+ is more resistant to acidification than I_{Ca}. Here an extended voltage range of the ramp protocol reveals IRK^+ as well as I_{Ca}. To isolate I_{Ca}, 20 mM TEA-Cl was added to the saline in these experiments. Under this condition the region of negative slope conductance can be mainly attributable to I_{Ca}, although it is known that 20 mM TEA-Cl does not suppress completely the outward rectifier K^+ current. I_{Ca} was activated at the potentials positive to -44 mV, peaked at around -17 mV, and the peak amplitude was 200 pA approximately. After superfusion of 20 mM Na-acetate, the peak amplitude of I_{Ca} was reduced to approximately 50%, while IRK^+ remained unchanged. From earlier studies based on simultaneous recordings of $[pH]_i$ and I_{Ca} in single cells, it can be concluded that a 50% reduction in I_{Ca} corresponds to a 0.3 pH unit acidification (see Fig. 6 in Dixon *et al.* [8]).

To lower intracellular pH below levels obtainable with Na-acetate superfusion and below those where I_{Ca} suppression could be used to estimate acidification, recordings were

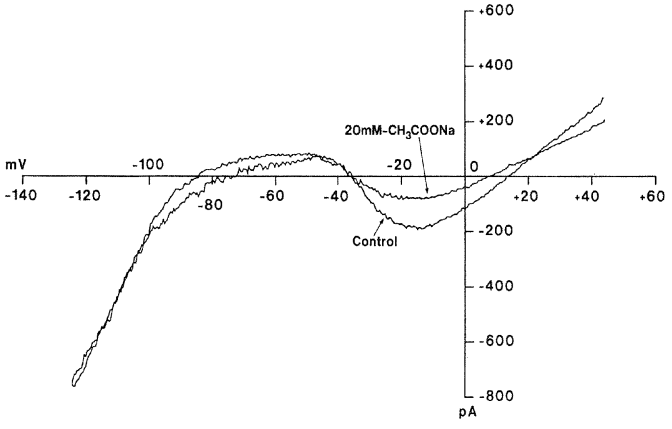

FIG. 3. Reduction of I_{Ca} by 20 mM Na-acetate. A voltage- ramp from -124 mV to $+46$ mV was applied to the cell. This revealed IRK^+ and a high-voltage activated, sustained calcium current, I_{Ca}. The peak current of I_{Ca} at -17 mV was reduced to about 50% in 20 mM Na-acetate, whereas IRK^+ was changed minimally.

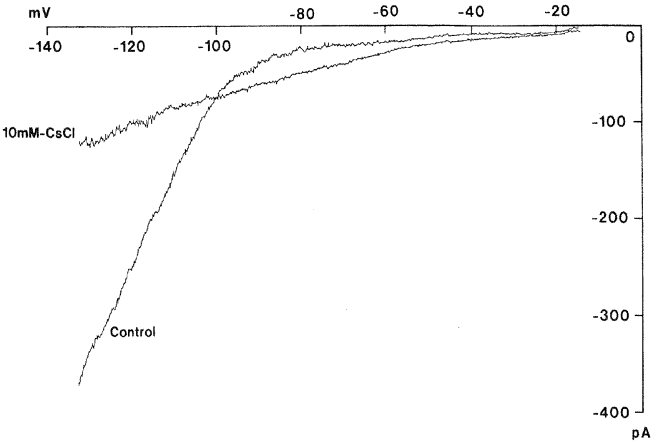

FIG. 4. Recording of IRK^+ with a pipette solution adjusted to pH 6.0. The pH of the pipette solution was adjusted at 6.0 in the absence of HEPES and was used for whole-cell recording. At this intracellular pH, IRK^+ was still present and activated at about -90 mV. This inward current activated at hyperpolarized potentials was blocked by 10 mM CsCl.

made using pipette solutions with the pH adjusted to 6.0. Even under these conditions IRK^+ was still easily recorded. Figure 4 shows the current recorded in a cell using a pH 6.0 pipette solution (control). The average amplitude of IRK^+ measured when the pipette solution pH was 6.0 was 644 ± 95 pA (n=6) at -134 mV. This was slightly smaller than that measured with the pH 7.4 (see Figures 2 and 3, for a example). In confirmation of the presence of IRK^+ at $[pH]_i$ of 6.0, Figure 4 demonstrates that there was a CsCl-sensitive current of almost 300 pA at -134 mV.

Effect of intracellular alkalinization

A saline containing 20 mM NH_4Cl was superfused in order to alkalinize horizontal cells. In NH_4Cl saline the peak current of IRK^+ at -134 mV was enhanced by $246 \pm 50\%$ (n=24). Figure 5A shows records obtained in control and in 20 mM NH_4Cl reveals an enhancement of the current at -134 mV from 840 pA to 1800 pA. This figure also illustrates that upon washout of NH_4Cl IRK^+ returned only to control despite intracellular acidification. In many cells the activation voltage for IRK^+ was shifted closer to zero mV in NH_4Cl. Hagiwara and Takahashi [10] showed that the activation voltage of anomalous rectifier currents depends on the difference between the membrane potential and the equilibrium potential for potassium ions. The shift in activation voltage observed with NH_4Cl suggests that NH_4^+ ions likely permeate IRK^+ channels [10].

To test whether NH_4Cl enhanced IRK^+ by alkalinization, the effect of NH_4Cl on IRK^+ was recorded with pipette solutions containing high amounts of pH-buffer. When the pipette solution contained 25 mM HEPES, 20 mM NH_4Cl enhanced IRK^+ by only $22.7 \pm 20.6\%$ (n=19) at -134 mV, much less than when a pH-unbuffered pipette solution was used. Figure 5B shows results from one cell exposed to NH_4Cl with 25 mM HEPES in the pipette solution.

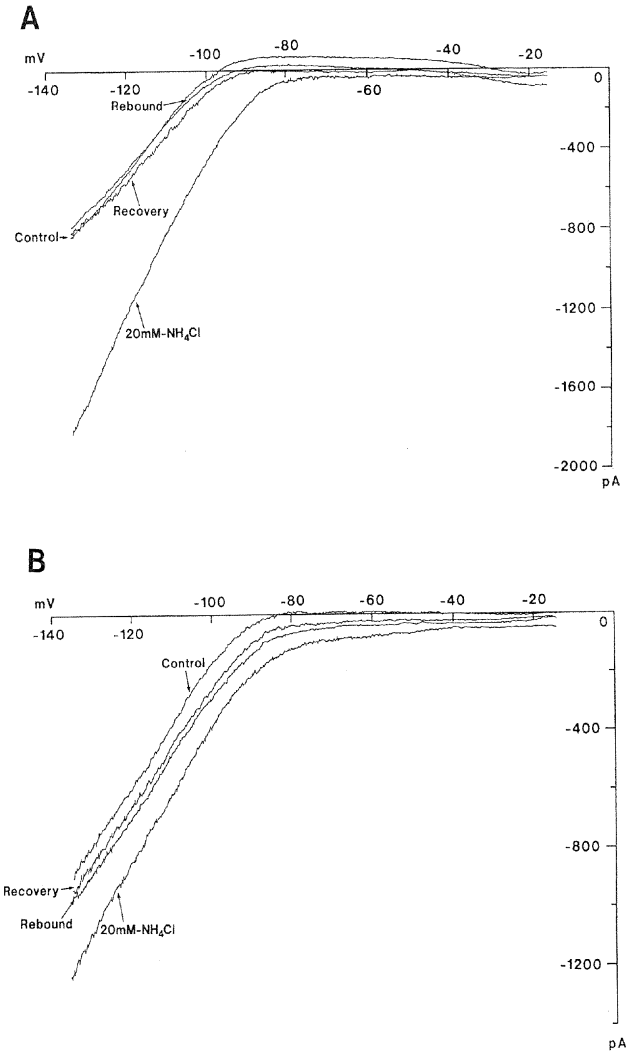

FIG. 5. Effect of 20 mM NH_4Cl on IRK^+. When NH_4Cl containing saline was superfused, the peak current at −134 mV was markedly increased (about 120%)(A). However, during the rebound acidification phase that follows the washout of NH_4Cl IRK^+ was not suppressed below control levels. In B, a pipette solution with 25 mM HEPES was used. The increase of the peak current caused by the superfusion of NH_4Cl was much smaller than in A.

The results presented here are consistent with the hypothesis that acidification below 7.4 minimally affects IRK^+. However, alkalinization above 7.4 significantly enhances this current. To further test this hypothesis we adjusted the pH of intracellular pipette solution to 7.8 and then observed how IRK^+ was altered by the alkalinization and rebound acidification produced by NH_4Cl. Figure 6 shows an example of the NH_4Cl effect on IRK^+ starting from an internal pH 7.8. During application of NH_4Cl IRK^+ was increased from 985 pA to 2000 pA at −134 mV. At this elevated pipette solution pH, IRK^+ had a peak amplitude of 1080±196 pA (n=8) at −134 mV. During washout, when [pH]i would be expected to be rebounding below resting pH, IRK^+ decreased to 650 pA at −134 mV (680±86pA, n=8).

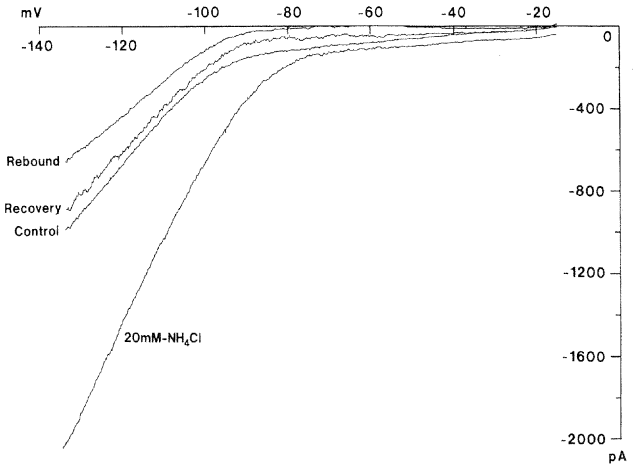

FIG. 6. Effect of 20 mM NH_4Cl on IRK^+ at an intracellular pH of 7.8. The pH of the pipette solution, in the absence of HEPES was adjusted to 7.8. When 20 mM NH_4Cl containing saline was superfused, the peak current was increased by about 100%. Following the washout of NH_4Cl, the IRK^+ was suppressed below control levels. More than 10 minutes after NH_4Cl washout, IRK^+ returned to control levels.

After recovering, IRK^+ returned to 920 pA at −134 mV (955 ±205 pA, n=8). Both the initial enhancement and suppression were observed in all 8 cells recorded with a pipette solution pH of 7.8.

DISCUSSION

Modulation of IRK^+ by change of $[pH]_i$

The present study demonstrates that IRK^+ in retinal horizontal cells is modified by changes of intracellular proton concentration. Specifically, IRK^+ is enhanced by intracellular alkalinization, but resistant to intracellular acidification below the normal resting pH of 7.4 [19]. The pH sensitivity of IRK^+ differs from that of I_{Ca} in these same cells. Takahashi *et al.* [20] reported that, in the same preparation, when the $[pH]_i$ was acidified below 7.4, I_{Ca} was suppressed significantly. In contrast, intracellular alkalinization of the cells caused only a small increase of I_{Ca}. Although both IRK^+ and I_{Ca} are voltage-sensitive channels, these results suggest that protons modify either the gating or conductances of these two channels differently.

In other cell types, pH sensitivity of voltage-gated K^+ currents has been reported. Moody and Hagiwara [15] reported that the inward rectifier in immature starfish oocytes was blocked by intracellular protons. A titration curve for the proton effect on the inward rectifier current was calculated for pH's between 7.09 and 5.9. The Hill coefficient and K_i were 3 and pH 6.4, respectively. Their titration curve showed that the inward rectifier current was saturated above pH 7.2. In these oocytes, $[pH]_i$ was measured at 7.23 when the bath was at pH 7.8. In our experiments, the mean amplitudes measured with a patch pipette solution pH of 6.0, 7.4 and 7.8 were at 644 pA, 795 pA and 1080 pA, respectively. We never observed the saturation of IRK^+ with alkali-

nization during the course of our experiments, indicating that the K_i for IRK^+ in horizontal cells is much more alkaline than that for oocytes. This suggests that the mechanism by which IRK^+ is modulated by $[pH]_i$ is different between these animal species.

It has also been reported that IRK^+ of skeletal muscle [3] and of ventricular cells [12] is blocked by intracellular acidification. Furthermore, delayed rectifier K^+ channels [23], Na^+ channels [5] and Ca^{2+} channels [11, 20, 22] have been shown to be sensitive to $[pH]_i$. It is now generally accepted that histidine residue(s) at intracellular domains in amino acid sequences composing the ion channels are the most likely sites for titration by protons. Since the Ki's for modulation by $[pH]_i$ differ from channel to channel as well as from preparation to preparation, other modulatory sites or different sequences or structures surrounding the histidine residues might be responsible for the observed variations. The exact mechanism(s) by which ionic channels are modulated via intracellular protons remains to be solved.

Is IRK^+ modulated via physiological changes of $[pH]_i$?

Because IRK^+ is reduced only slightly for acidifications below resting $[pH]_i$, this current will only be modified by intracellular alkalinization.

In general, mechanisms that alter $[pH]_i$ include: (1) changes in metabolic production of CO_2 and lactic acid, (2) changes in free intracellular calcium concentration that regulates the release of protons from internal sites, and (3) changes in the net transport of acid through ligand- and voltage-dependent channels. Typically increased metabolism, increased internal calcium and activation of GABA receptors or glutamate receptors under physiological conditions produce intracellular acidification that range from 0.05 to 0.4 pH units [6, 7]. The circumstances under which one would expect an alkalinization include: (1) a reduction in metabolic activity, such as occurs with cooling; (2) reduced intracellular calcium levels, perhaps due to increases in the activity of a sodium/calcium exchanger; and finally, (3) during prolonged reduction of transmitter release, that might occur if extracellular calcium levels were depleted. Examples of alkalinizations due to these mechanisms have yet to be documented in horizontal cells. In conclusion, the present results show that IRK^+ is relatively stable over the physiological range of $[pH]_i$, unlike I_{Ca} [8, 20].

Would glutamate modulate IRK^+ via intracellular acidification?

Glutamate, the photoreceptor transmitter, reduces I_{Ca} in catfish horizontal cells via intracellular acidification [8]. Kaneko and Tachibana [13] reported that glutamate blocked IRK^+ in horizontal cells dissociated from goldfish retina. Before the results from the present study were obtained, it was a logical possibility that glutamate might be suppressing IRK^+ by acidification. However, this blocking action of IRK^+ by glutamate seems not to be mediated by pH. Our results show that the 0.3 pH unit acidification caused by 100 μM-glutamate (see Fig. 4 in Dixon *et al.* [8]) would not explain the suppression of IRK^+. Therefore, it is very likely that the anomalous rectifier channels must be modulated by glutamate through pH-independent mechanisms [13], perhaps through metabotropic glutamate receptors.

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