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Regulation of Cl^-–HCO_3^- exchangers by cAMP-dependent protein kinase in adult rat hippocampal CA1 neurons

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The contributions of HCO_3^--dependent, DIDS-sensitive mechanisms to the maintenance of steady-state pH_i, and the regulation of their activities by cAMP-dependent protein kinase (PKA), were investigated in CA1 neurons with the H^+-sensitive fluorophore, BCECF. The addition of HCO_3^-/CO_2 to neurons with 'low' ($pH_i \leqslant 7.20$) and 'high' ($pH_i > 7.20$) initial pH_i values under Hepes-buffered conditions, increased and decreased steady-state pH_i, respectively. Conversely, under HCO_3^-/CO_2-buffered conditions, DIDS caused pH_i to decrease and increase in neurons with low and high initial pH_i values, respectively. In the presence, but not the absence, of HCO_3^-, the PKA inhibitor Rp-adenosine-3′,5′-cyclic monophosphorothioate (Rp-cAMPS; 50 μM) evoked DIDS-sensitive increases and decreases in pH_i in neurons with low and high initial pH_i values, respectively. In contrast, in neurons with low initial pH_i values, activation of PKA with the Sp isomer of cAMPS (Sp-cAMPS; 25 μM) elicited increases in pH_i that were smaller in the presence than in the absence of HCO_3^-, whereas in neurons with high initial pH_i values, Sp-cAMPS-evoked rises in pH_i were larger in the presence than in the absence of HCO_3^-; the differences between the effects of Sp-cAMPS on pH_i under the different buffering conditions were attenuated by DIDS. Consistent with the possibility that changes in the activities of HCO_3^--dependent, DIDS-sensitive mechanisms contribute to the steady-state pH_i changes evoked by the PKA modulators, in neurons with initial pH_i values $\leqslant 7.20$, Rp-cAMPS concurrently inhibited Na^+-independent Cl^-–HCO_3^- exchange and stimulated Na^+-dependent Cl^-–HCO_3^- exchange; in contrast, Sp-cAMPS concurrently stimulated Na^+-independent Cl^-–HCO_3^- exchange and inhibited Na^+-dependent Cl^-–HCO_3^- exchange. Data from a limited number of neurons with initial pH_i values > 7.20 suggested that the directions of the reciprocal changes in anion exchange activities (inhibition or stimulation) evoked by Rp- and Sp-cAMPS may be opposite in cells with low *vs.* high resting pH_i values. Taken together, the results indicate that the effects of modulating PKA activity on steady-state pH_i in rat CA1 neurons under HCO_3^-/CO_2-buffered conditions reflect not only changes in Na^+–H^+ exchange activity but also changes in Na^+-dependent and Na^+-independent Cl^-–HCO_3^- exchange activity that, in turn, may be dependent upon the initial pH_i.

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Intracellular pH (pH_i) is an important determinant of neuronal function and yet, compared to non-neuronal cell types, relatively little information is available concerning either the mechanisms that act to regulate pH_i in mammalian central neurons or the factors that control their activities. In rat hippocampal neurons, the majority of studies have focused on the participation of Na^+–H^+ exchange in acid extrusion and the maintenance of steady-state pH_i; these studies have also established that Na^+–H^+ exchange activity can be modulated by pH_i, pH_o, temperature, intracellular second messengers and pathophysiological events such as anoxia (Raley-Susman *et al.* 1991; Baxter & Church, 1996; Bevensee *et al.* 1996; Smith *et al.* 1998; Diarra *et al.* 1999; Sheldon & Church, 2002). By comparison, relatively little is known about the roles of HCO_3^--dependent mechanisms in pH_i regulation in this cell type. Although Na^+-dependent and Na^+-independent Cl^-–HCO_3^- exchangers have been identified in rat CA1 neurons (Kopito *et al.* 1989; Raley-Susman *et al.* 1993; Grichtchenko *et al.* 2001*b*), and the former has been shown to contribute to DIDS-sensitive acid extrusion (Schwiening & Boron, 1994; Baxter & Church, 1996), neither their roles in the maintenance of steady-state pH_i nor the possibility that their activities might be regulated by second messengers have been systematically addressed. The paucity of information available concerning both the function and the regulation of the activities of Cl^-–HCO_3^- exchangers in mammalian central neurons is surprising, not only because they are important determinants of pH_i in other nucleated cell types (Roos & Boron, 1981; Vaughan-

Jones, 1986; Olsnes *et al.* 1987; Boyarsky *et al.* 1988; Cassel *et al.* 1988; Ganz *et al.* 1989; Kikeri *et al.* 1990; Tønnessen *et al.* 1990; Kramhøft *et al.* 1994; Strazzabosco *et al.* 1997; Leem *et al.* 1999) but also because they play important roles in morphogenesis, cytoskeletal reorganization and other cellular properties (e.g. Phillips & Baltz, 1999; Schwab, 2001). It has also been established that the activities of Cl^-–HCO_3^- exchangers in a variety of cell types can be regulated by intracellular second messengers, with consequent effects on cellular function (e.g. Boron *et al.* 1978; Reuss, 1987; Vigne *et al.* 1988; Green *et al.* 1990; Ludt *et al.* 1991; Pucéat *et al.* 1998).

In the present study, we initially assessed the contribution of HCO_3^--dependent, DIDS-sensitive mechanisms to the maintenance of steady-state pH_i in our preparation of acutely dissociated adult rat hippocampal CA1 neurons (see Schwiening & Boron, 1994; Bevensee *et al.* 1996). Next, in the knowledge that activation of cAMP-dependent protein kinase (PKA) under nominally HCO_3^-/CO_2-free, Hepes-buffered conditions increases pH_i in rat CA1 neurons by stimulating Na^+–H^+ exchange (Smith *et al.* 1998), we asked whether PKA is involved in the control of pH_i under physiological conditions, that is in the presence of HCO_3^-/CO_2. In light of the results, which indicated that the modulation of PKA activity leads to HCO_3^--dependent, DIDS-sensitive changes in steady-state pH_i, we examined the regulation by PKA of the activities of the Na^+-dependent and Na^+-independent Cl^-–HCO_3^- exchangers which contribute to acid and alkali extrusion, respectively, in rat CA1 neurons.

Portions of this work have been presented in abstract form (Brett & Church, 1998; Kelly *et al.* 2000).

METHODS

Cell preparation

All procedures conformed to guidelines established by the Canadian Council on Animal Care and were approved by The University of British Columbia Animal Care Committee.

Acutely dissociated CA1 neurons were prepared as previously described (Smith *et al.* 1998). In brief, male Wistar rats (200–260 g) were anaesthetized with 3 % halothane in air and rapidly decapitated. Transverse hippocampal slices (450 μm) were cut and allowed to recover for 1 h in HCO_3^-/CO_2-buffered saline (see below). To isolate CA1 neurons, slices were enzymatically digested for 30 min in HCO_3^-/CO_2-buffered saline containing 1.5 mg ml^{-1} protease type XIV (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada); the CA1 regions were then microdissected and triturated with fire-polished Pasteur pipettes of diminishing tip diameters. The triturated suspension was deposited onto a glass coverslip mounted in a perfusion chamber to form the floor of the chamber and neurons were allowed to adhere for 30 min, during which time they were loaded with fluorophore (see below).

Solutions and chemicals

The standard nominally HCO_3^-/CO_2-free perfusion medium contained (mM): NaCl 136.5, KCl 3, $CaCl_2$ 2, NaH_2PO_4 1.5, $MgSO_4$ 1.5, D-glucose 17.5 and Hepes 10; and was titrated to the appropriate temperature-corrected pH with 10 M NaOH. In standard HCO_3^-/CO_2-buffered media, Hepes was isosmotically replaced by NaCl and solutions contained either 19.5 mM (37 °C) or 29 mM (room temperature, RT; 20–22 °C) $NaHCO_3$, by equimolar substitution for NaCl, together with the constituents listed above; pH measured in the recording chamber was 7.35 after equilibration with 5 % CO_2–95 % O_2. Solutions containing 20 mM NH_4Cl were prepared by equimolar substitution for NaCl. For Na^+-free HCO_3^-/CO_2-buffered media, NaH_2PO_4 was omitted and Na^+ salts were replaced with the appropriate choline salts. When external Cl^- was omitted, it was replaced isosmotically with gluconate. The Na^+-free, Cl^--free HCO_3^-/CO_2-buffered medium contained (mM): choline base (aqueous) 127, choline HCO_3^- 19.5, D-gluconic acid 127, potassium gluconate 3, hemicalcium gluconate 4, $MgSO_4$ 1.5 and D-glucose 17.5; pH 7.35 after equilibration with 5 % CO_2–95 % O_2. The HCO_3^-/CO_2 solutions employed to impose internal alkali loads contained twice the [$NaHCO_3$] as standard HCO_3^-/CO_2-buffered media, by isosmotic substitution for NaCl, and were equilibrated with 10 % CO_2–90 % O_2 (pH 7.35).

Test compounds were obtained from Sigma-Aldrich Canada Ltd with the exceptions of 2′,5′-dideoxyadenosine (DDA; Biomol Research Laboratories Inc., Plymouth Meeting, PA, USA) and the Sp- and Rp- isomers of adenosine-3′,5′-cyclic monophosphorothioate (Sp- and Rp-cAMPS, respectively; Biolog Life Science Institute, La Jolla, CA, USA). Test compounds were applied by superfusion. Stock solutions of DIDS were prepared on the day of the experiment, immediately prior to dilution with experimental media to the working concentration (200 μM).

Recording techniques

Neurons were loaded with the acetoxymethyl ester form of 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF, 2 μM for 15 min; Molecular Probes Inc., Eugene, OR, USA) and were then superfused at 2 ml min^{-1} for 15 min with the initial experimental solution at the appropriate experimental temperature prior to the start of an experiment. Experiments were performed at 37 °C unless otherwise noted in the text.

The dual-excitation ratio method was used to estimate pH_i, employing a digital fluorescence ratio imaging system (Atto Instruments Inc., Rockville, MD, USA). Details of the methods employed have been presented previously (Baxter & Church, 1996; Smith *et al.* 1998; Sheldon & Church, 2002). In brief, fluorescence emissions measured at 520 nm were detected by an intensified charge-coupled device camera and collected from regions of interest placed on individual neuronal somata. Raw emission intensity data at each excitation wavelength (488 and 452 nm) were corrected for background fluorescence prior to calculation of the ratio. Ratio pairs were acquired at 1–12 s intervals. Analysis was restricted to those neurons able to retain BCECF, as judged by the stability of the fluorescence emission intensity recorded during excitation at 452 nm, throughout the course of an experiment (see Bevensee *et al.* 1996). To reduce photobleaching of the fluorophore and cell damage, the output of the 100 W mercury arc lamp was attenuated electronically, neutral density filters were placed in the light path and a high-speed shutter was employed to limit light exposure to periods required for data acquisition.

The one point high-[K^+]/nigericin technique was employed to convert background-corrected BCECF emission intensity ratios (BI_{488}/BI_{452}) into pH_i values (see Boyarsky *et al.* 1996). Parameters employed in the calculation of pH_i values were derived from non-

linear least squares regression fits to background-subtracted ratio *vs.* pH data which, in turn, were obtained in full calibration experiments (see Baxter & Church, 1996). For the seventeen full calibration experiments utilized in analysing all data, the mean values for $R_{n,max}$ (the maximum obtainable value for the normalized ratio), $R_{n,min}$ (the minimum obtainable value for the normalized ratio) and pK_a (the −log of the dissociation constant of BCECF) were (means ± S.E.M.) 1.88 ± 0.04, 0.41 ± 0.02 and 7.16 ± 0.02, respectively. These values were not dependent on the temperature at which the calibration was conducted. To limit cross-contamination by nigericin, perfusion lines were replaced and the imaging chamber was decontaminated after each experiment by soaking in ethanol and then in 20% Decon 75 (BDH Inc., Toronto, ON, Canada), as described by Leem *et al.* (1999).

Experimental procedures and data analysis

The effects of changes in perfusate composition and pharmacological treatments were examined on steady-state pH_i and/or rates of pH_i recovery from internal acid loads (imposed by the NH_4^+ prepulse technique) or alkaline loads (imposed by exposure to and subsequent removal of medium containing high concentrations of HCO_3^- and CO_2 at a constant pH_o).

As detailed in the Results, the effects of modulating PKA activity on steady-state pH_i and Cl^-–HCO_3^- exchanger activities were dependent on the initial pH_i of a neuron prior to a test treatment (also see Bevensee *et al.* 1996). In any given series of experiments, neurons were classified as 'low' ($pH_i \leqslant 7.20$) or 'high' ($pH_i > 7.20$) pH_i neurons on the basis of a least squares regression fit to data points relating the changes in pH_i evoked on the transition from a Hepes- to a HCO_3^-/CO_2-buffered medium at a constant pH_o (7.35) to the initial pH_i values under Hepes-buffered conditions (Fig. 1*B*; also see Schwiening & Boron, 1994; Smith *et al.* 1998).

In each experiment in which rates of pH_i recovery were examined, two consecutive acid or alkali loads were imposed, the first being employed to calculate control rates of pH_i recovery for a given neuron and the second being performed under the influence of a test treatment. The consistency of rates of pH_i recovery following two consecutive acid or alkali loads imposed in the absence of a test treatment was established in control experiments (data not shown; see Smith *et al.* 1998; Sheldon & Church, 2002). Full details of the methods employed in analysing data obtained in acid load recovery experiments have been presented previously (Baxter & Church, 1996; Smith *et al.* 1998). In brief, the recovery of pH_i following an NH_4^+ prepulse was fitted to a single exponential function and the first derivative of this function was used to determine the rate of pH_i change (dpH_i/dt). Instantaneous rates of pH_i recovery under control and test conditions were then plotted against absolute pH_i values, compared statistically at corresponding values of pH_i, and the data points were fitted by weighted non-linear least squares regression ($r^2 \geqslant 0.90$ in all cases). In addition, at each corresponding absolute value of pH_i, the percentage difference between the control rate of pH_i recovery and the rate of pH_i recovery under the influence of a test treatment was calculated and the mean of the resultant differences was employed to describe the overall effect of a test treatment on the rate of pH_i recovery. Similar procedures were employed to quantify rates of pH_i recovery from internal alkali loads, except that the recovery was fitted to a single exponential decay function; analysis was then identical to the procedures used to analyse rates of pH_i recovery from acid loads.

Data are reported as means ± S.E.M., with the accompanying n value referring to the number of neurons from which data were obtained. Statistical comparisons were performed with Student's two-tailed t tests (paired or unpaired, as appropriate); significance was assumed at the 5% level.

RESULTS

Contribution of HCO_3^--dependent, DIDS-sensitive mechanisms to the maintenance of pH_i

Initially, we examined the effects on pH_i of exposing neurons originally perfused with Hepes-buffered medium to a solution buffered with HCO_3^-/CO_2 at a constant pH_o (7.35). As previously described (Schwiening & Boron, 1994; Smith *et al.* 1998), switching to a HCO_3^-/CO_2-containing medium caused pH_i to increase and decrease in neurons with low ($\leqslant 7.20$; $n = 28$) and high (> 7.20; $n = 11$) initial pH_i values, respectively, in Hepes-buffered medium (Fig. 1*A* and *B*). The increases and decreases in pH_i occasioned by the addition of HCO_3^- in neurons with low and high pH_i values, respectively, under nominally HCO_3^--free conditions are consistent with the shift from a bimodal to a unimodal distribution of steady-state pH_i values that occurs in rat CA1 neurons upon exposure to HCO_3^-/CO_2 (see Bevensee *et al.* 1996; Smith *et al.* 1998). They are also consistent with previous reports (Raley-Susman *et al.* 1993; Schwiening & Boron, 1994; Baxter & Church, 1996) that Na^+-independent and Na^+-dependent Cl^-–HCO_3^- exchange contribute to base and acid extrusion, respectively, from rat hippocampal neurons. The former transport mechanism is most active at high pH_i values whereas the latter is most active at low pH_i values and, in many cell types, the two exchangers act in concert to determine steady-state pH_i (e.g. Olsnes *et al.* 1987; Boyarsky *et al.* 1988; Cassel *et al.* 1988; Ganz *et al.* 1989; Green *et al.* 1990; Kikeri *et al.* 1990; Mugharbil *et al.* 1990; Tønnessen *et al.* 1990; Kramhøft *et al.* 1994; Leem *et al.* 1999). The fact that, depending on the initial pH_i in Hepes-buffered medium, pH_i could increase or decrease upon exposure to HCO_3^-, suggested the possibilities that activation of Na^+-independent Cl^-–HCO_3^- exchange might contribute to the HCO_3^--induced fall in pH_i in cells which, in Hepes, had high resting pH_i values, and that activation of Na^+-dependent Cl^-–HCO_3^- exchange might contribute to the HCO_3^--induced rise in pH_i in cells which, in Hepes, had low resting pH_i values. In this regard, we (Baxter & Church, 1996) and others (Schwiening & Boron, 1994) have failed to uncover a contribution from Na^+–HCO_3^- cotransport to the regulation of pH_i in rat CA1 neuron somata (also see Schmitt *et al.* 2000).

Both Na^+-dependent and Na^+-independent Cl^-–HCO_3^- exchangers are typically sensitive to DIDS. We have shown previously that DIDS fails to affect steady-state pH_i in rat CA1 neurons under nominally HCO_3^-/CO_2-free, Hepes-buffered conditions (Smith *et al.* 1998). However, applied in the presence of HCO_3^-/CO_2, DIDS elicited decreases and increases in pH_i in neurons with low ($n = 26$) and high ($n = 11$) initial pH_i values, respectively (Fig. 1*C*); in seven

additional cells with pH_i values in the range 7.05–7.31, DIDS failed to affect steady-state pH_i. The DIDS-evoked changes in pH_i were plotted against the pH_i values measured prior to its addition and a least squares fit to the data points had a positive slope and an *x*-intercept at pH_i 7.17 (Fig. 1*D*), which is similar to that obtained from the regression line relating the changes in pH_i evoked by the addition of HCO_3^- to initial pH_i values under Hepes-buffered conditions (pH_i 7.21; Fig. 1*B*). Thus, the addition of DIDS under HCO_3^-/CO_2-buffered conditions elicited qualitatively opposite changes in pH_i to those seen upon the transition from a Hepes- to a HCO_3^-/CO_2-buffered medium at a constant pH_o.

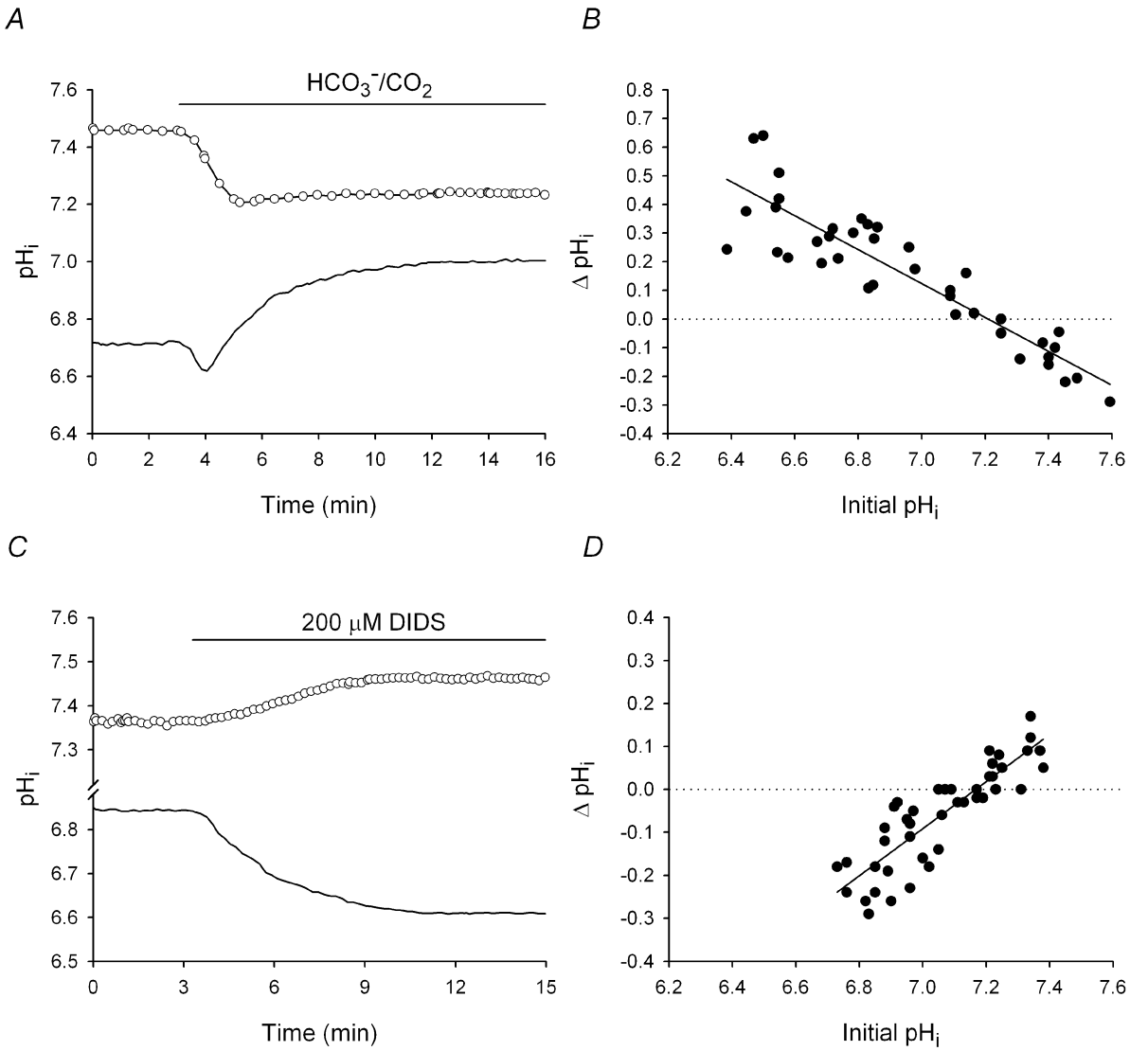

Figure 1. Effects on pH_i of the transition from Hepes- to HCO_3^-/CO_2-buffered medium, and the addition of DIDS to HCO_3^-/CO_2-buffered medium

A, a neuron perfused with a Hepes-buffered medium (pH 7.35) had a low initial pH_i (continuous line). Upon exposure to HCO_3^-/CO_2-buffered medium at a constant pH_o, pH_i decreased transiently and then increased to a new steady-state level. In contrast, a different neuron with a high initial pH_i in Hepes-buffered medium (○) exhibited an internal acidification upon exposure to HCO_3^-/CO_2. *B*, the changes in pH_i (ΔpH_i) evoked by the addition of HCO_3^-/CO_2 plotted against initial pH_i values in Hepes-buffered medium ($n = 39$). A linear least squares regression fit to the data points ($r^2 = 0.84$) had a negative slope and intersected the abscissa at pH_i 7.21. *C*, in a neuron with a low initial pH_i value under HCO_3^-/CO_2-buffered conditions at pH_o 7.35 (continuous line), DIDS caused pH_i to decrease to a new steady-state level. In contrast, DIDS caused pH_i to rise in a different neuron with a high initial pH_i (○). *D*, ΔpH_i elicited by 200 μM DIDS applied under HCO_3^-/CO_2-buffered conditions plotted against initial pH_i values ($n = 44$). A linear least squares regression fit to the data points ($r^2 = 0.75$) had a similar *x*-intercept (pH_i 7.17) but an opposite slope to the fit representing the pH_i response to HCO_3^-/CO_2 application.

Taken together, the findings are consistent with previous reports (Schwiening & Boron, 1994; Baxter & Church, 1996; Bevensee *et al.* 1996; Smith *et al.* 1998) that HCO_3^--dependent, DIDS-sensitive mechanism(s) contribute to the regulation of pH_i in rat hippocampal neurons.

Effect of modulating PKA activity on pH_i under HCO_3^-/CO_2-buffered conditions

In rat CA1 neurons under nominally HCO_3^-/CO_2-free conditions at 37 °C, activation of the cAMP/PKA second messenger pathway produces an alkaline shift in the pH_i dependence of Na^+–H^+ exchange and thereby increases steady-state pH_i; in contrast, inhibiting the pathway fails to affect either Na^+–H^+ exchange activity or steady-state pH_i (Smith *et al.* 1998). In light of the above findings, we therefore examined whether modulating the activity of the cAMP/PKA pathway evokes DIDS-sensitive changes in pH_i in the presence of HCO_3^-/CO_2.

In agreement with the results of Smith *et al.* (1998), inhibiting PKA with 50 μM Rp-cAMPS under Hepes-buffered conditions failed to affect pH_i in 10 and seven neurons with initial pH_i values $\leqslant 7.20$ and > 7.20, respectively (data not shown). In contrast, under HCO_3^-/CO_2-buffered conditions, Rp-cAMPS elicited changes in pH_i that were dependent on the resting pH_i prior to the modulation of PKA activity. Thus, as illustrated in Fig. 2*A*, Rp-cAMPS increased pH_i in neurons with low initial pH_i values and decreased pH_i in neurons with high initial pH_i values. When the Rp-cAMPS-evoked changes in pH_i were plotted against initial pH_i values (Fig. 2*B*), the regression fit to the data points had a negative slope and a similar *x*-intercept (pH_i 7.26) as the fit representing the change in pH_i that occurred on the transition from a Hepes- to a HCO_3^-/CO_2-buffered medium (Fig. 1*B*). The changes in pH_i evoked by Rp-cAMPS in the presence of HCO_3^- were attenuated by pre-treatment with DIDS (Fig. 2*B*) and were mimicked by the adenylate cyclase inhibitor DDA (100 μM; $n = 11$; data not shown). Next, the effects of activating PKA were examined. As illustrated in Fig. 2*C*, under Hepes-buffered conditions 25 μM Sp-cAMPS elicited a 0.21 ± 0.03 ($n = 8$) pH unit increase in pH_i in neurons with initial pH_i values $\leqslant 7.20$, which is in agreement with the findings of Smith *et al.* (1998). However in the presence of HCO_3^-, 25 μM Sp-cAMPS caused a 0.11 ± 0.01 pH unit increase in pH_i in nine neurons also with low initial pH_i values ($P < 0.02$ for the difference to the change in pH_i observed in the absence of HCO_3^-). The effect of HCO_3^- to decrease the magnitude of the rise in pH_i evoked by Sp-cAMPS in low pH_i neurons was inhibited by DIDS; in six neurons pre-treated with DIDS for 10–15 min, 25 μM Sp-cAMPS evoked a larger increase in pH_i (0.23 ± 0.03 pH units) than observed in the absence of the stilbene ($P < 0.01$). In contrast to results obtained in low pH_i neurons, the rise in pH_i evoked by 25 μM Sp-cAMPS in cells with initial pH_i values > 7.20 was smaller in the absence ($a\ 0.10 \pm 0.02$ pH unit increase; $n = 7$) than in the presence ($a\ 0.26 \pm 0.02$ pH unit increase; $n = 6$) of HCO_3^-/CO_2 ($P < 0.02$; Fig. 2*C*). These data are summarized in Fig. 2*D*, which illustrates that Sp-cAMPS tended to evoke larger rises in pH_i under Hepes- than under HCO_3^-/CO_2-buffered conditions in neurons with initial pH_i values $\leqslant 7.20$ whereas, in neurons with initial pH_i values > 7.20, the opposite was true. The effects of Sp-cAMPS on steady-state pH_i were mimicked by the adenylate cyclase activator forskolin (25 μM; $n = 11$ and 23 under HCO_3^--containing and nominally HCO_3^--free conditions, respectively) whereas an inactive analogue of forskolin, 1′,9′-dideoxyforskolin (25 μM; $n = 6$ in each case), failed to influence pH_i (data not shown; also see Smith *et al.* 1998).

To further explore the effects of PKA modulators on steady-state pH_i under HCO_3^-/CO_2-buffered conditions, experiments were repeated in the absence of external Na^+, under which condition forward Na^+–H^+ and Na^+-dependent Cl^-–HCO_3^- exchange are blocked. Similar to observations made in the presence of Na^+_o, 50 μM Rp-cAMPS applied under Na^+_o-free conditions evoked a rise in pH_i of 0.15 ± 0.03 pH units in nine neurons with low initial pH_i values and a fall in pH_i of 0.18 ± 0.06 pH units in four neurons with high initial pH_i values (Fig. 2*E*); these effects were abolished by pre-treatment with DIDS ($n = 4$, low pH_i neurons and $n = 3$, high pH_i neurons). In contrast, 25 μM Sp-cAMPS elicited a 0.07 ± 0.01 pH unit decrease in pH_i in five neurons with initial pH_i values $\leqslant 7.20$ and a 0.12 ± 0.03 pH unit increase in pH_i in four neurons with initial pH_i values > 7.20 (Fig. 2*F*). Thus, the directions of the pH_i changes evoked by Sp-cAMPS under Na^+_o-free conditions in low and high pH_i neurons were opposite to and the same as those observed in the presence of Na^+_o, respectively (see Fig. 2*C*). The pH_i changes evoked by Sp-cAMPS in low ($n = 3$) and high ($n = 5$) pH_i cells in the absence of external Na^+ were abolished by pre-treatment with DIDS. Finally, applied under Na^+_o- and Cl^-_o-free conditions (where all known acid/base transporters in rat CA1 neurons are inactive), neither 50 μM Rp-cAMPS ($n = 4$ low pH_i neurons and $n = 2$ high pH_i neurons) nor 25 μM Sp-cAMPS ($n = 5$ low pH_i neurons and $n = 2$ high pH_i neurons) significantly affected steady-state pH_i.

PKA-dependent modulation of Na^+-independent and Na^+-dependent Cl^-–HCO_3^- exchange

The above findings indicate that modulating the activity of the cAMP/PKA second messenger pathway leads to HCO_3^--dependent, DIDS-sensitive changes in steady-state pH_i, the magnitudes and/or directions of which depend on the initial pH_i. Insofar as Na^+-dependent and Na^+-independent Cl^-–HCO_3^- exchange are the only HCO_3^--dependent, DIDS-sensitive mechanisms found to date to participate in pH_i regulation in rat CA1 neurons (see Introduction), together with the fact that these

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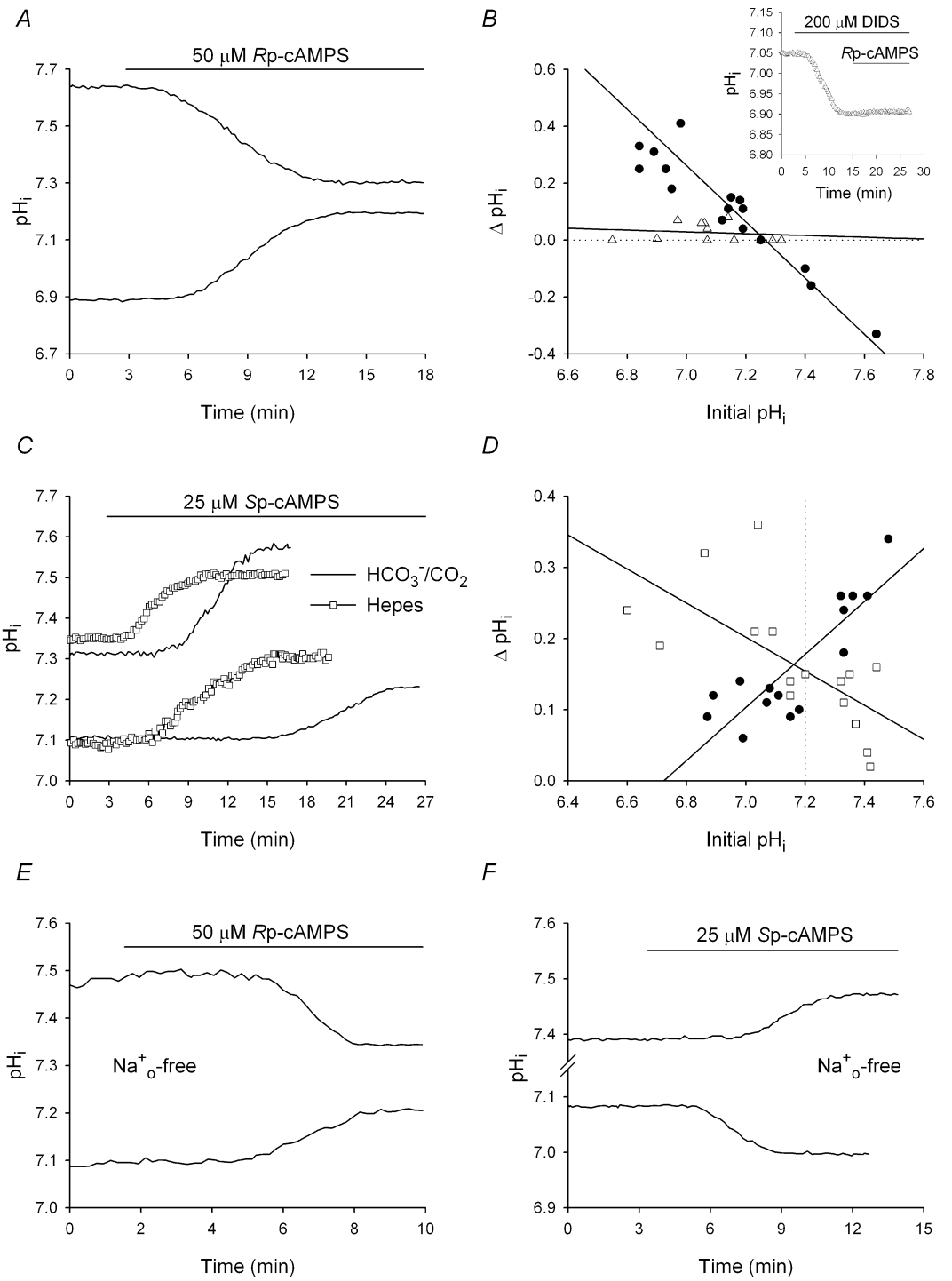

Figure 2. Effects of Rp- and Sp-cAMPS on pH_i

A, Rp-cAMPS, applied under HCO_3^-/CO_2-buffered conditions (pH_o 7.35) for the period indicated by the bar above the traces, evoked increases and decreases in pH_i in two different neurons with low and high initial pH_i values, respectively, prior to the addition of the PKA inhibitor. *B*, the changes in pH_i (ΔpH_i) elicited by 50 μM Rp-cAMPS under HCO_3^-/CO_2-buffered control conditions (●) or following 10–15 min pre-treatment with 200 μM DIDS (△) were plotted against pH_i values measured prior to the addition of the PKA inhibitor and each set of data points was fitted by least squares regression ($r^2 \geqslant 0.80$). The changes in pH_i evoked by Rp-cAMPS were dependent upon the initial pH_i and, as illustrated in the inset, were attenuated by DIDS. *C*, four different neurons, two with similarly low initial pH_i values and two with similarly high initial pH_i values, were exposed to 25 μM Sp-cAMPS under HCO_3^-/CO_2- (continuous line) or Hepes- (□) buffered conditions. In neurons with initial pH_i values $\leqslant$ 7.20, Sp-cAMPS evoked a greater increase in pH_i under Hepes- than under HCO_3^-/CO_2-buffered conditions; in contrast, in neurons with initial pH_i values > 7.20, Sp-cAMPS evoked a smaller increase in pH_i under Hepes- than under HCO_3^-/CO_2-buffered conditions. *D*, ΔpH_i elicited by 25 μM

Table 1. Changes in pH_i evoked by modulating PKA activity under HCO_3^-/CO_2-buffered conditions and potential underlying alterations in Cl^--HCO_3^- exchanger activities

Initial pH_i	Treatment	Observed change in pH_i	Potential alteration in Cl^--HCO_3^- exchange activity		
			Na^+-independent		Na^+-dependent
⩽ 7.20	Rp-cAMPS	↑	↓	and/or	↑
	Sp-cAMPS	↓*	↑	and/or	↓
> 7.20	Rp-cAMPS	↓	↑	and/or	↓
	Sp-cAMPS	↑*	↓	and/or	↑

Rp-cAMPS and Sp-cAMPS were applied at 50 μM and 25 μM, respectively. *Relative to the change in pH_i evoked by Sp-cAMPS under nominally HCO_3^-/CO_2-free, Hepes-buffered conditions.

exchangers are active over a wide, overlapping range of physiologically relevant pH_i values (e.g. Boron *et al.* 1979; Olsnes *et al.* 1987; Boyarsky *et al.* 1988; Cassel *et al.* 1988; Kikeri *et al.* 1990; Tønnessen *et al.* 1990; Kramhøft *et al.* 1994; Strazzabosco *et al.* 1997; Leem *et al.* 1999), four possibilities could account for the observed effects of modulating PKA activity on steady-state pH_i under HCO_3^-/CO_2-buffered conditions (Table 1). In brief, HCO_3^--dependent, DIDS-sensitive increases in pH_i may in part reflect an increased rate of acid extrusion via Na^+-dependent anion exchange and/or a decreased rate of acid loading via Na^+-independent anion exchange. Conversely, stimulation of Na^+-independent Cl^-–HCO_3^- exchange and/or inhibition of Na^+-dependent Cl^-–HCO_3^- exchange may contribute to HCO_3^--dependent, DIDS-sensitive decreases in pH_i. Therefore, in the third series of experiments, we examined the effects of modulating PKA activity on Na^+-independent and Na^+-dependent Cl^-–HCO_3^- exchange.

Na^+-independent Cl^-–HCO_3^- exchange

Forward Na^+-independent Cl^-–HCO_3^- exchange. Consistent with the participation of Na^+-independent Cl^-–HCO_3^- exchange in the recovery of pH_i from base loading in CA1 neurons, pH_i recovery from internal alkali loads was inhibited by DIDS ($n = 6$) or under Cl^-_o-free conditions ($n = 8$) (Fig. 3*A*), and could proceed in the absence of Na^+_o ($n = 7$; see Figs 3*D* and 4*C*). Alkali loads were then imposed in the absence and presence of 50 μM Rp-cAMPS or 25 μM Sp-cAMPS; all neurons in these experiments had pH_i values ⩽ 7.20 prior to modulating PKA activity. The overall rate of pH_i recovery from alkali loads decreased ~4.5-fold in the presence compared to the absence of Rp-cAMPS in 14 cells under Na^+_o-containing conditions (Fig. 3*B*). As shown in Fig. 3*C*, Rp-cAMPS reduced rates of pH_i recovery ($P < 0.05$ at all absolute values of pH_i) and shifted the pH_i dependence of the rate of pH_i recovery in the alkaline direction; similar effects were observed in the absence of Na^+_o ($n = 7$; Fig. 3*D*). In contrast to the effects of Rp-cAMPS, activation of PKA with Sp-cAMPS increased rates of pH_i recovery from alkali loads under Na^+_o-containing ($n = 9$; Fig. 4*A* and *B*) and Na^+_o-free ($n = 5$; Fig. 4*C*) conditions.

Reverse Na^+-independent Cl^-–HCO_3^- exchange. In cultured rat hippocampal neurons, acute exposure to Cl^--free, HCO_3^--buffered medium elicits an internal alkalinization that is blocked by DIDS and persists in the absence of external Na^+, which is consistent with reverse Na^+-independent Cl^-–HCO_3^- exchange activity (Baxter & Church, 1996; also see Boyarsky *et al.* 1988; Ganz *et al.* 1989; Raley-Susman *et al.* 1993). In the present study in acutely isolated adult neurons, the acute removal of Cl^-_o elicited a reversible 0.13 ± 0.01 pH unit rise in pH_i ($n = 31$ low pH_i neurons; Fig. 5) that was dependent on HCO_3^- and blocked by DIDS ($n = 8$ in each case; data not shown). Also in neurons with pH_i values ⩽ 7.20 prior to the removal of Cl^-_o, exposure to Cl^--free medium in the presence of 50 μM Rp-cAMPS or 25 μM Sp-cAMPS evoked increases in pH_i of 0.07 ± 0.01 ($n = 4$; Fig. 5*A*) and 0.42 ± 0.07 pH units ($n = 6$; Fig. 5*B*), respectively ($P < 0.05$ in each case, compared to the alkalinizations observed in the absence of a test treatment). The increases in pH_i observed during the acute removal of Cl^-_o in the presence of Rp- or Sp-cAMPS were abolished by DIDS ($n = 4$ in each case; see Fig. 5*B*) and, as shown in Fig. 5*C*, differences in pH_i values prior to the introduction of Cl^--free medium could not account for the observed effects of the PKA modulators on the DIDS-sensitive alkalinizations induced in low pH_i cells by Cl^-_o removal.

Sp-cAMPS in the nominal absence (□) or presence (●) of HCO_3^-/CO_2 were plotted against initial values of pH_i and a regression line was fitted to each set of data points. The vertical dotted line represents the division between 'low' (initial pH_i ⩽ 7.20) and 'high' (initial pH_i > 7.20) pH_i neurons. *E*, the same experiment as that shown in *A*, but conducted in the absence of external Na^+. Rp-cAMPS evoked an increase and a decrease in pH_i in two different neurons with low and high initial pH_i values, respectively. *F*, under Na^+_o-free, HCO_3^--buffered conditions, the addition of 25 μM Sp-cAMPS caused pH_i to increase in a neuron with a high initial pH_i, and pH_i to decrease in a different neuron with a low initial pH_i.

Na^+-dependent Cl^-–HCO_3^- exchange

pH_i recovery from acid loads. Under HCO_3^-/CO_2-buffered conditions at 37 °C, the effect of activating PKA on rates of pH_i recovery from acid loads will reflect not only potential changes in Na^+-dependent Cl^-–HCO_3^- exchange activity but also changes in the activity of the acid-extruding Na^+–H^+ exchanger (Smith *et al.* 1998). However, Na^+–H^+ exchange in rat hippocampal neurons is insensitive to amiloride, amiloride derivatives and guanidinium compounds (Raley-Susman *et al.* 1991; Schwiening & Boron, 1994; Baxter & Church, 1996; Bevensee *et al.* 1996), precluding the use of selective pharmacological inhibitors of Na^+–H^+ exchange to isolate the effect of a test treatment on Na^+-dependent

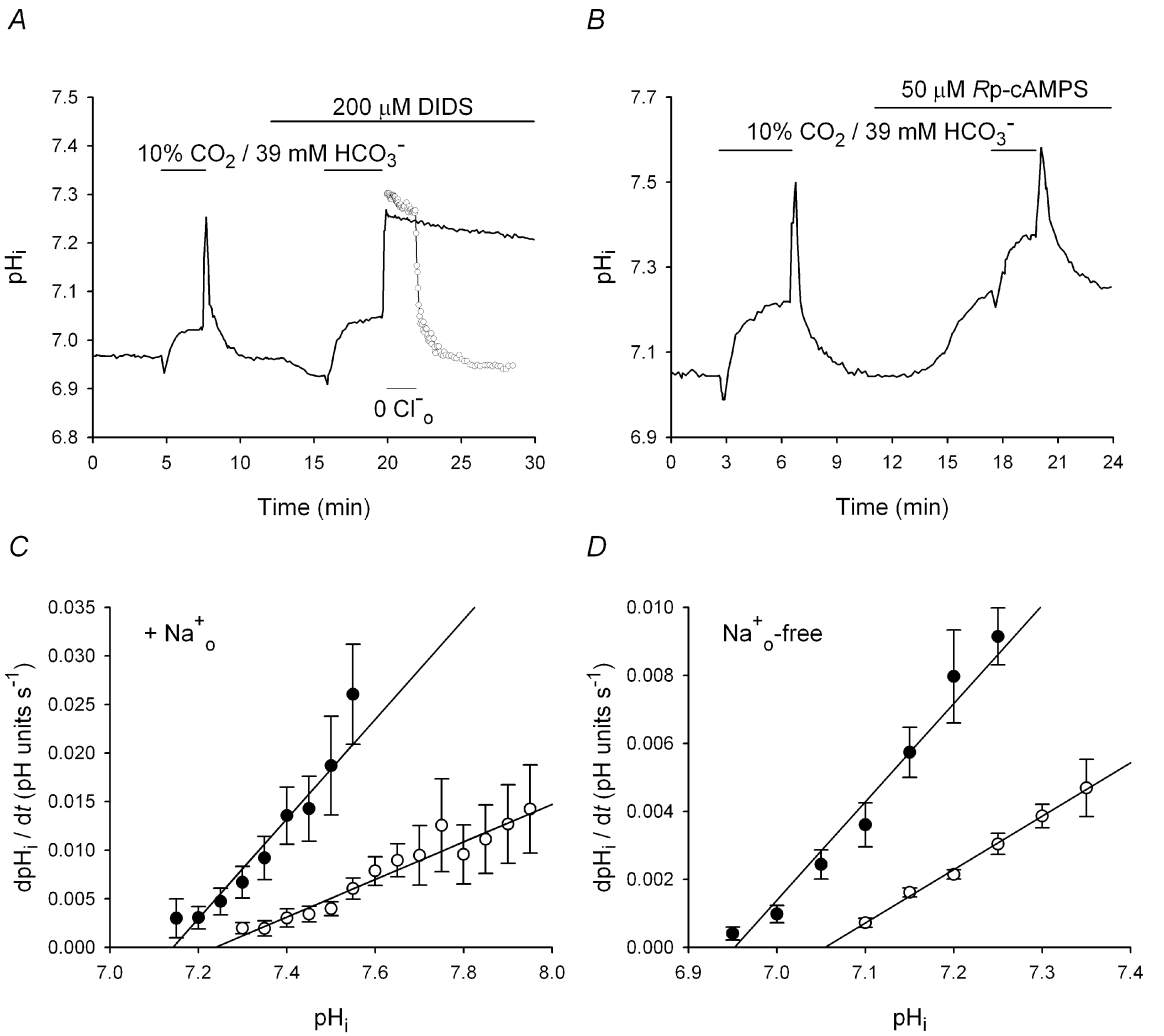

Figure 3. Effects of Rp-cAMPS on pH_i recovery from base loading in the presence and absence of Na^+_o

All data were obtained from neurons with initial pH_i values ⩽ 7.20 under HCO_3^-/CO_2-buffered conditions. Alkali loads were imposed by applying and withdrawing a 10 % CO_2/39 mM HCO_3^- solution, as indicated by the short bars above the traces in *A* and *B*. *A*, following an initial alkali load, pH_i was allowed to recover and a second alkali load was imposed in the presence of DIDS (applied for the period indicated by the long bar above the trace). Superimposed is a record (○) obtained from a different cell that was exposed to Cl^--free medium (applied for the period indicated by the short bar beneath the trace) at the peak of the second alkali load (the first part of the record from this experiment has been omitted for clarity). *B*, after recovery from an initial alkali load imposed under control conditions, the neuron was exposed to 50 μM Rp-cAMPS. Rp-cAMPS evoked a rise in pH_i (see Fig. 2*A*) and a second alkali load was then imposed; the rate of pH_i recovery was decreased in the presence of Rp-cAMPS. *C*, mean rates of pH_i recovery following alkali loads imposed in the absence (●) and presence (○) of 50 μM Rp-cAMPS plotted against absolute values of pH_i. Data points were obtained from 14 experiments of the type illustrated in *B*; error bars represent S.E.M. *D*, mean rates of pH_i recovery from alkali loads obtained in the absence (●) and presence (○) of 50 μM Rp-cAMPS under Na^+_o-free conditions plotted against absolute values of pH_i. Data points were obtained from seven experiments of the type illustrated in *B*, except in the absence of external Na^+; error bars represent S.E.M.

Cl^-–HCO_3^- exchange. Nevertheless, Na^+–H^+ exchange activity in rat hippocampal neurons is markedly inhibited at RT, compared to 37 °C, and under reduced temperature conditions Na^+-dependent Cl^-–HCO_3^- exchange becomes the dominant mechanism whereby pH_i recovers from internal acid loads (Baxter & Church, 1996; Sheldon & Church, 2002). In agreement with the latter reports, in control experiments conducted as part of the present study, rates of pH_i recovery from acid loads imposed in acutely isolated adult rat CA1 neurons at 37 °C were similar in the presence and nominal absence of HCO_3^-/CO_2 ($(4.88 \pm 0.65) \times 10^{-3}$ pH units s^{-1}, $n = 4$ and $(4.66 \pm 0.94) \times 10^{-3}$ pH units s^{-1}, $n = 5$, respectively, measured at a common test pH_i of 6.65). In contrast, at RT, not only were rates of pH_i recovery significantly faster in the presence ($(2.48 \pm 0.40) \times 10^{-3}$ pH units s^{-1}, $n = 4$) than in the absence ($(0.82 \pm 0.40) \times 10^{-3}$ pH units s^{-1}, $n = 5$) of HCO_3^-/CO_2 but also the higher rates of pH_i recovery observed in the presence of HCO_3^- were reduced to $0.98 \pm 0.60 \times 10^{-3}$ pH units s^{-1} ($n = 3$) in the presence of DIDS. Therefore, to assess the effects of modulating PKA activity on Na^+-dependent Cl^-–HCO_3^- exchange in relative isolation, the majority of experiments in this series were performed at RT.

As previously described (Smith *et al.* 1998), under Hepes-buffered conditions at 37 °C, 25 μM Sp-cAMPS increased the overall rate of pH_i recovery from acid loads by 148 ± 16 % ($n = 5$; not shown); however at 20–22 °C, an increase of only 2 ± 10 % ($n = 9$) was observed (Fig. 6*A* and *B*). These findings are consistent with suggestions (see above) that Na^+–H^+ exchange plays only a limited role in the recovery of pH_i from acid loads at RT. In contrast to the lack of effect of Sp-cAMPS under Hepes-buffered conditions at RT, under HCO_3^-/CO_2-buffered conditions at RT Sp-cAMPS evoked a 58 ± 3 % ($n = 6$) decrease in the overall rate of pH_i recovery in neurons with initial pH_i values $\leqslant 7.20$ (Fig. 6*C*). The effect of Sp-cAMPS to decrease rates of pH_i recovery from acid loads imposed in the presence of HCO_3^- at RT in low pH_i neurons was blocked by DIDS (the overall change in the rate of pH_i recovery evoked by Sp-cAMPS in the presence of DIDS was a 16 ± 7 % increase; $n = 9$). In contrast to results obtained in low pH_i neurons, in neurons with initial pH_i values > 7.20, Sp-cAMPS evoked an overall increase of 385 ± 42 % ($n = 6$) in rates of pH_i recovery from acid loads imposed under HCO_3^-/CO_2-buffered conditions at RT (Fig. 6*D*); pre-treatment with DIDS attenuated the increase to 26 ± 7 % ($n = 6$). The results are consistent

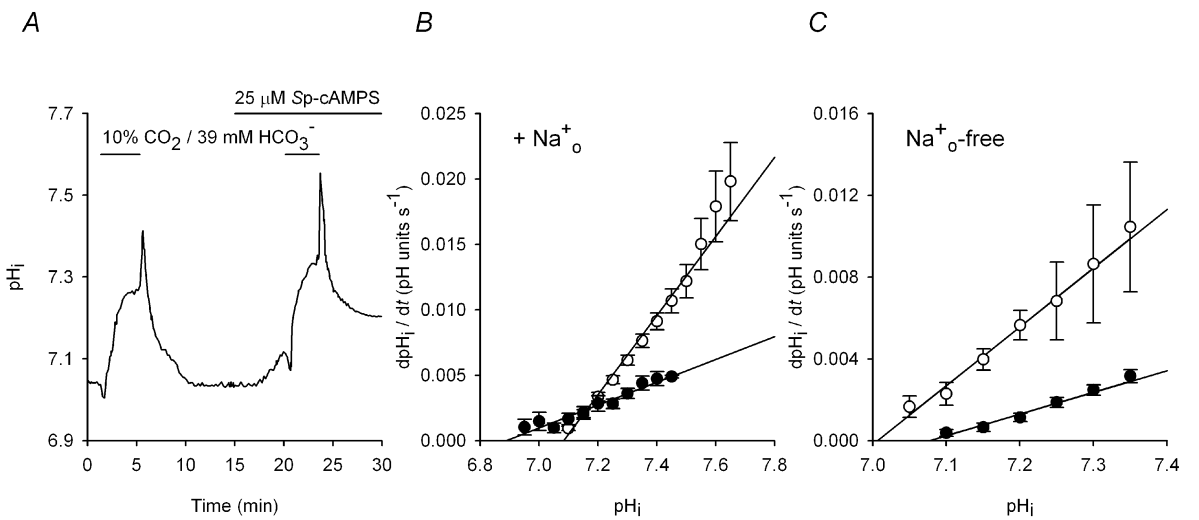

Figure 4. Effects of Sp-cAMPS on pH_i recovery from base loading in the presence and absence of Na^+_o

All data were obtained from neurons with initial pH_i values $\leqslant 7.20$ under HCO_3^-/CO_2-buffered conditions. *A*, after the recovery of pH_i from a control alkali load imposed under Na^+_o-containing conditions, 25 μM Sp-cAMPS evoked a slow increase in steady-state pH_i (see Fig. 2*C*). A second alkali load was then imposed and pH_i recovered at a faster rate, and to a new steady-state level, in the continued presence of Sp-cAMPS. *B*, the pH_i dependence of pH_i recovery under Na^+_o-containing conditions in the absence (●) and presence (○) of 25 μM Sp-cAMPS. Data points were obtained from nine experiments of the type illustrated in *A*; error bars represent S.E.M. The alkaline shift in the pH_i-dependence of pH_i recovery from alkali loads evoked by Sp-cAMPS reflects the increase in pH_i elicited by Sp-cAMPS in the presence of Na^+_o in neurons with resting pH_i values $\leqslant 7.20$. *C*, mean rates of pH_i recovery from base loading in the absence (●) and presence (○) of 25 μM Sp-cAMPS under Na^+_o-free conditions plotted against absolute values of pH_i. Data points were obtained from five experiments of the type illustrated in *A*, except in the absence of external Na^+. The acidic shift in the pH_i-dependence of pH_i recovery from alkali loads in the presence of Sp-cAMPS reflects the decrease in pH_i elicited by Sp-cAMPS in the absence of Na^+_o in neurons with resting pH_i values $\leqslant 7.20$ (see Fig. 2*F*).

with the possibility (Table 1) that Sp-cAMPS inhibits Na^+-dependent Cl^-–HCO_3^- exchange in neurons with initial pH_i values ⩽ 7.20 and also suggest that, in neurons with initial pH_i values > 7.20, activation of PKA may stimulate Na^+-dependent Cl^-–HCO_3^- exchange.

Next, acid loads were imposed in the absence and presence of 50 μM Rp-cAMPS. As observed previously at 37 °C (Smith *et al.* 1998), at RT application of Rp-cAMPS failed to significantly change rates of pH_i recovery from acid loads imposed in low pH_i neurons in the absence of HCO_3^- (n = 10; data not shown). In contrast, under HCO_3^-/CO_2-buffered conditions, Rp-cAMPS increased overall rates of pH_i recovery in neurons with initial pH_i values ⩽ 7.20 by 239 ± 39 % (n = 12; Fig. 7*A* and *B*) and 193 ± 33 % (n = 8; Fig. 7*C* and *D*) at RT and at 37 °C, respectively. The effect of Rp-cAMPS to increase rates of pH_i recovery from acid loads imposed in low pH_i neurons under HCO_3^-/CO_2-buffered conditions was attenuated by pre-treatment with DIDS; the overall increases in rates of pH_i recovery evoked by Rp-cAMPS in the presence of DIDS at RT and at 37 °C were 19 ± 21 % (n = 3) and 11 ± 14 % (n = 4), respectively. Thus, in low pH_i neurons, Rp-cAMPS increased rates of pH_i recovery from acid loads in a HCO_3^--dependent, DIDS-sensitive manner. The results are consistent with the possibility (Table 1) that inhibition of PKA stimulates Na^+-dependent Cl^-–HCO_3^- exchange in neurons with initial pH_i values ⩽ 7.20. No neurons with initial pH_i values > 7.20 were encountered in this series of experiments.

Effects of depleting internal Cl^-. In a second set of experiments designed to examine the effects of PKA modulators on Na^+-dependent Cl^-–HCO_3^- exchange, we employed a technique in which Na^+-dependent Cl^-–HCO_3^- exchange is repeatedly activated in the absence of external Cl^-, resulting in the gradual depletion of Cl^-_i and inhibition of the exchange mechanism (see Schwiening & Boron, 1994). We reasoned that Na^+-dependent Cl^-–HCO_3^- exchange would 'run-down' more quickly in the presence of PKA modulators that stimulate the exchange mechanism, whereas the opposite would be true for PKA modulators that inhibit the transporter. All neurons examined in these experiments had initial pH_i values ⩽ 7.20.

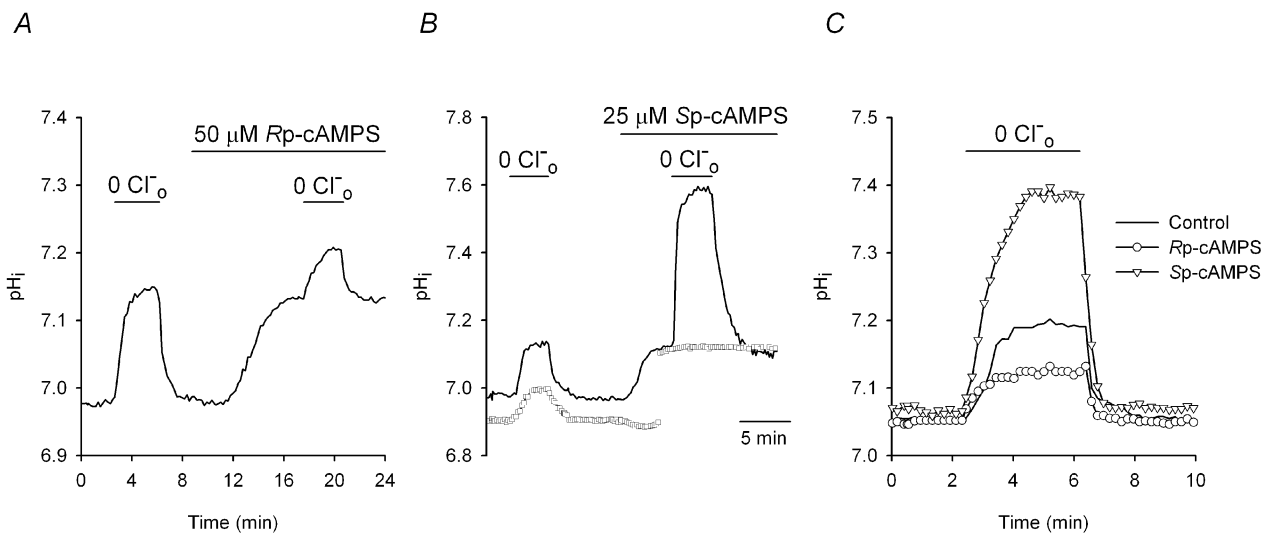

Figure 5. Effects of Rp- and Sp-cAMPS on changes in pH_i evoked by removal of Cl^-_o

All data were obtained from neurons with initial pH_i values ⩽ 7.20 under HCO_3^-/CO_2-buffered conditions. *A*, the acute removal of Cl^-_o (for the period indicated by the short bar above the trace) evoked an increase in pH_i which recovered upon the reintroduction of the anion. Rp-cAMPS (50 μM) was then applied and, after pH_i increased to a new steady-state level (see Fig. 2*A*), the removal of Cl^-_o evoked an increase in pH_i that was smaller than in the absence of the PKA inhibitor. Increases in pH_i evoked by the acute removal of Cl^-_o in the presence of a PKA modulator were measured as the difference between the maximum pH_i observed in the absence of Cl^-_o and the plateau pH_i value observed following the reintroduction of Cl^-_o in the continued presence of the PKA modulator. *B*, a neuron with a low initial pH_i (continuous line) was exposed to Cl^--free medium, which caused an increase in pH_i. Sp-cAMPS (25 μM) was then applied; the PKA activator evoked an increase in pH_i (see Fig. 2*C*) and subsequent exposure to Cl^--free medium caused a large internal alkalinization. Superimposed is a record (□) obtained from a different low pH_i neuron in which 25 μM Sp-cAMPS was co-applied with 200 μM DIDS; the gap in the trace represents a 5.5 min break in the record. The rise in pH_i observed during exposure to Cl^--free medium in the presence of Sp-cAMPS was blocked by DIDS. *C*, the increases in pH_i evoked by transient exposure to Cl^--free medium are shown in three different neurons with similar initial pH_i values under control conditions (continuous line) and following pre-treatment with 25 μM Sp-cAMPS (▽) or 50 μM Rp-cAMPS (○).

As illustrated in Fig. 8*A*, an initial application of HCO_3^-/CO_2 in the absence of external Cl^- elicited a transient acidification (due to CO_2 entry) followed by a 0.10 ± 0.02 pH unit ($n = 7$) increase in pH_i, which returned to the initial steady-state value upon the removal of HCO_3^-/CO_2. Second, third and fourth exposures to HCO_3^-/CO_2, in the continued absence of Cl^-_o, caused 0.08 ± 0.02, 0.06 ± 0.02 and 0.05 ± 0.02 pH unit increases in pH_i, respectively. Instantaneous rates of change in pH_i during each exposure to HCO_3^-/CO_2 were calculated at an absolute pH_i value of 7.00 and were then normalized to the mean rate of change in pH_i observed during the first application of HCO_3^-/CO_2 (Fig. 8*C*). The progressive decline in the normalized rates of alkalinization evoked by repeated HCO_3^-/CO_2 applications in the absence of Cl^-_o suggests that Na^+-dependent Cl^-–HCO_3^- exchange activity was declining progressively due to depletion of Cl^-_i (see Schwiening & Boron, 1994).

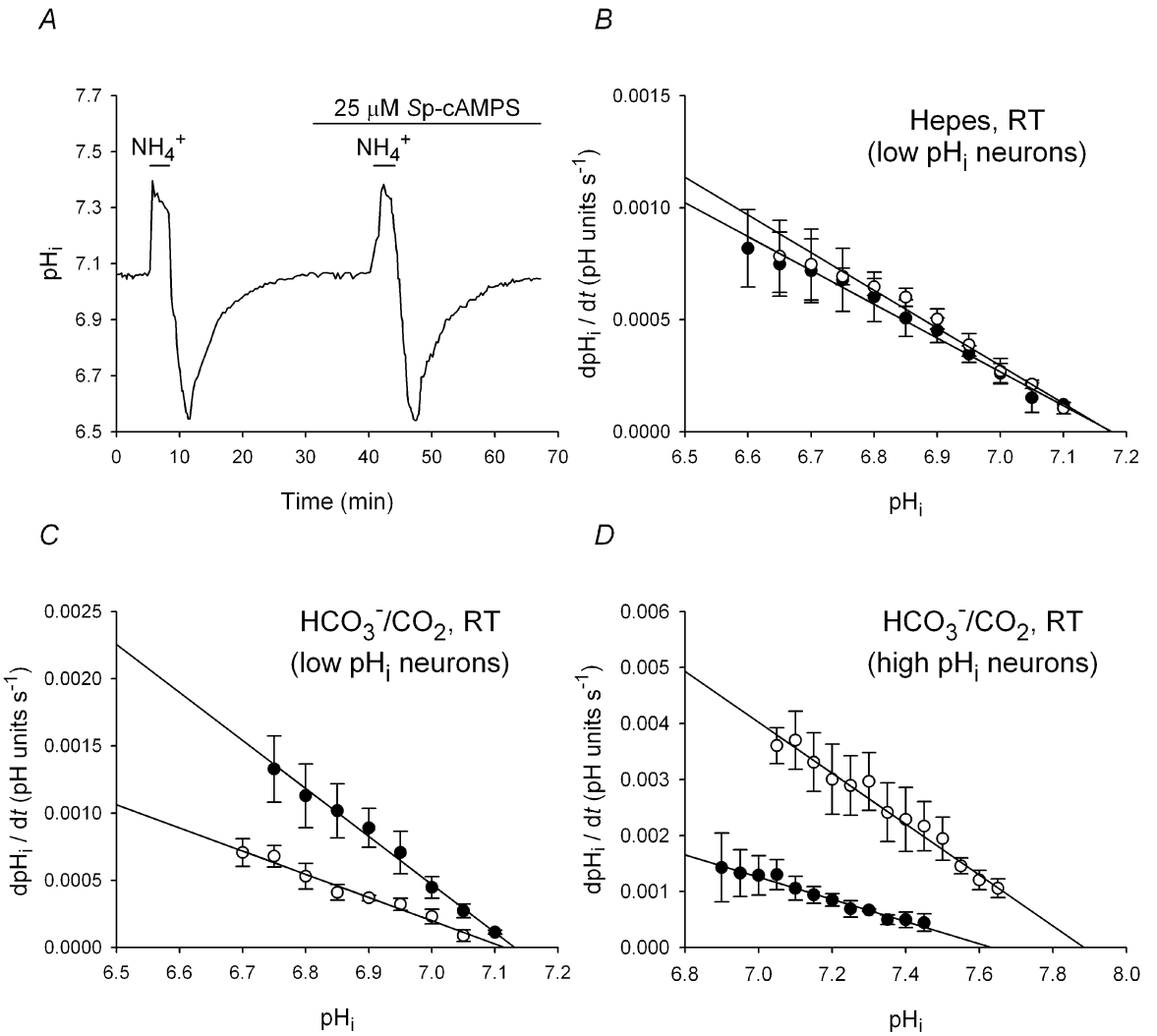

Figure 6. Effects of Sp-cAMPS on pH_i recovery from acid loads at room temperature

A, two consecutive acid loads, the second in the presence of 25 μM Sp-cAMPS, were imposed on a neuron with a low initial pH_i under Hepes-buffered conditions. Rates of pH_i recovery from both acid loads were similar and, in contrast to the effects of Sp-cAMPS under Hepes-buffered conditions at 37 °C (see Smith *et al.* 1998), pH_i failed to recover to a higher steady-state level in the presence of Sp-cAMPS. *B*, rates of pH_i recovery from acid loads performed in the absence (●) and presence (○) of 25 μM Sp-cAMPS plotted against absolute values of pH_i; data points were obtained from nine experiments of the type shown in *A* (error bars represent S.E.M.). Sp-cAMPS failed to significantly affect the rate of pH_i recovery at any absolute value of pH_i. *C*, the pH_i dependence of pH_i recovery under HCO_3^-/CO_2-buffered conditions at RT in the absence (●) and presence (○) of 25 μM Sp-cAMPS; data points were obtained from six experiments of the type shown in *A*, except in the presence of HCO_3^-/CO_2. Rates of pH_i recovery were significantly reduced in the presence of Sp-cAMPS at all absolute values of pH_i. *D*, rates of pH_i recovery from acid loads imposed on high pH_i neurons ($n = 6$) under HCO_3^-/CO_2-buffered conditions at RT in the absence (●) and presence (○) of 25 μM Sp-cAMPS plotted against absolute values of pH_i. In contrast to its effect in low pH_i neurons, Sp-cAMPS increased rates of pH_i recovery in neurons with high initial pH_i values.

Similar experiments were then performed in the presence of 50 μM Rp-cAMPS or 25 μM Sp-cAMPS ($n = 4$ in each case; see Fig. 8*B*), and normalized rates of change of pH_i were calculated at an absolute pH_i value of 7.00 during four consecutive exposures to HCO_3^-/CO_2 under each experimental condition. In the absence of a PKA modulator, the normalized rate of alkalinization became significantly decreased during the third exposure to HCO_3^-/CO_2 (Fig. 8*C*). However, in the presence of Rp-cAMPS, the normalized rate of alkalinization became significantly decreased during the second exposure to HCO_3^-/CO_2, suggesting that internal Cl^- was being depleted faster in the presence than in the absence of Rp-cAMPS. In contrast, normalized rates of alkalinization obtained in the presence of Sp-cAMPS failed to decline in response to four successive exposures to HCO_3^-/CO_2, suggesting that internal Cl^- was not being depleted. The results are consistent with those obtained in the acid load recovery experiments and further support the possibilities (Table 1) that inhibition and stimulation of PKA, increases and decreases, respectively, Na^+-dependent Cl^-–HCO_3^- exchange activity in rat CA1 neurons with initial pH_i values $\leqslant 7.20$.

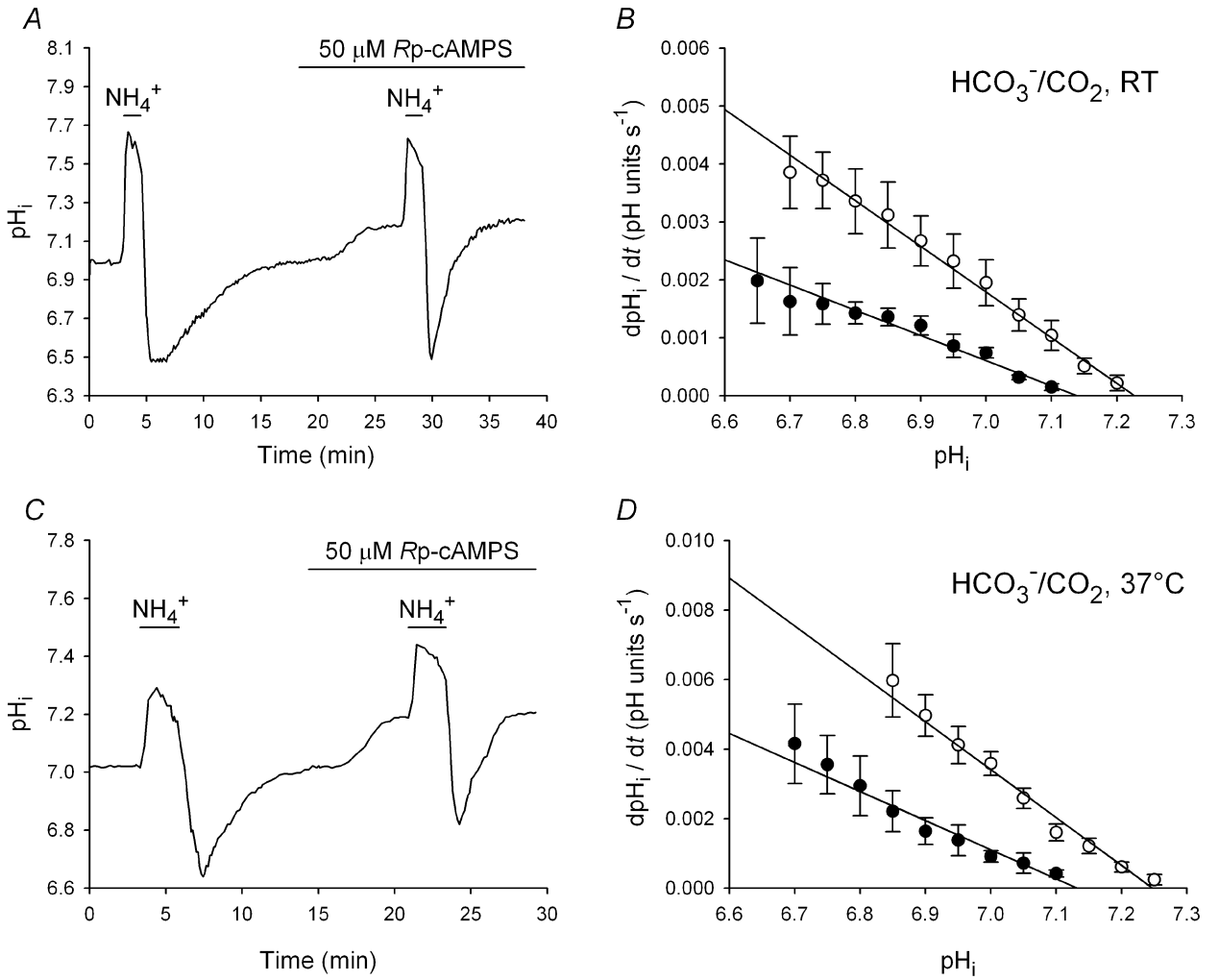

Figure 7. Effects of Rp-cAMPS on pH_i recovery from acid loads in low pH_i neurons

A, an acid load was imposed on a low pH_i neuron under HCO_3^-/CO_2-buffered conditions at RT and, after recovery of pH_i, 50 μM Rp-cAMPS was applied. Rp-cAMPS increased both steady-state pH_i and the rate of pH_i recovery from the second acid load. *B*, the pH_i dependence of the rate of pH_i recovery from acid loads imposed under HCO_3^-/CO_2-buffered conditions at RT in the absence (●) and presence (○) of 50 μM Rp-cAMPS; data points were obtained from 12 experiments of the type shown in *A*. *C*, the same experiment as shown in *A*, but conducted at 37 °C. *D*, the pH_i dependence of the rate of pH_i recovery from acid loads performed at 37 °C in the absence (●) and presence (○) of 50 μM Rp-cAMPS; data points were obtained from eight experiments of the type shown in *C*. In contrast to observations made in the nominal absence of HCO_3^-/CO_2, Rp-cAMPS applied to low pH_i neurons in the presence of HCO_3^-/CO_2 increased rates of pH_i recovery at all absolute values of pH_i and shifted the pH_i dependence of the rate of pH_i recovery in an alkaline direction.

DISCUSSION

The results of the study indicate that: (a) HCO_3^--dependent, DIDS-sensitive mechanisms are important determinants of pH_i in adult rat CA1 neurons at physiological pH_o; (b) inhibiting or activating PKA under HCO_3^-/CO_2-buffered conditions leads to DIDS-sensitive changes in pH_i, the directions and magnitudes of which are related to the initial pH_i of a neuron prior to the modulation of PKA activity; and (c) the effects of modulating PKA on steady-state pH_i in the presence of HCO_3^-/CO_2 may in part be mediated by alterations in Na^+-dependent and Na^+-independent Cl^-–HCO_3^- exchange activity (and, in the case of PKA activation, Na^+–H^+ exchange).

Steady-state pH_i in the presence of HCO_3^-/CO_2

It has previously been shown that Na^+-dependent Cl^-–HCO_3^- exchange contributes to acid extrusion from rat CA1 neurons and that activation of this mechanism underlies the sustained increases in pH_i that occur in low

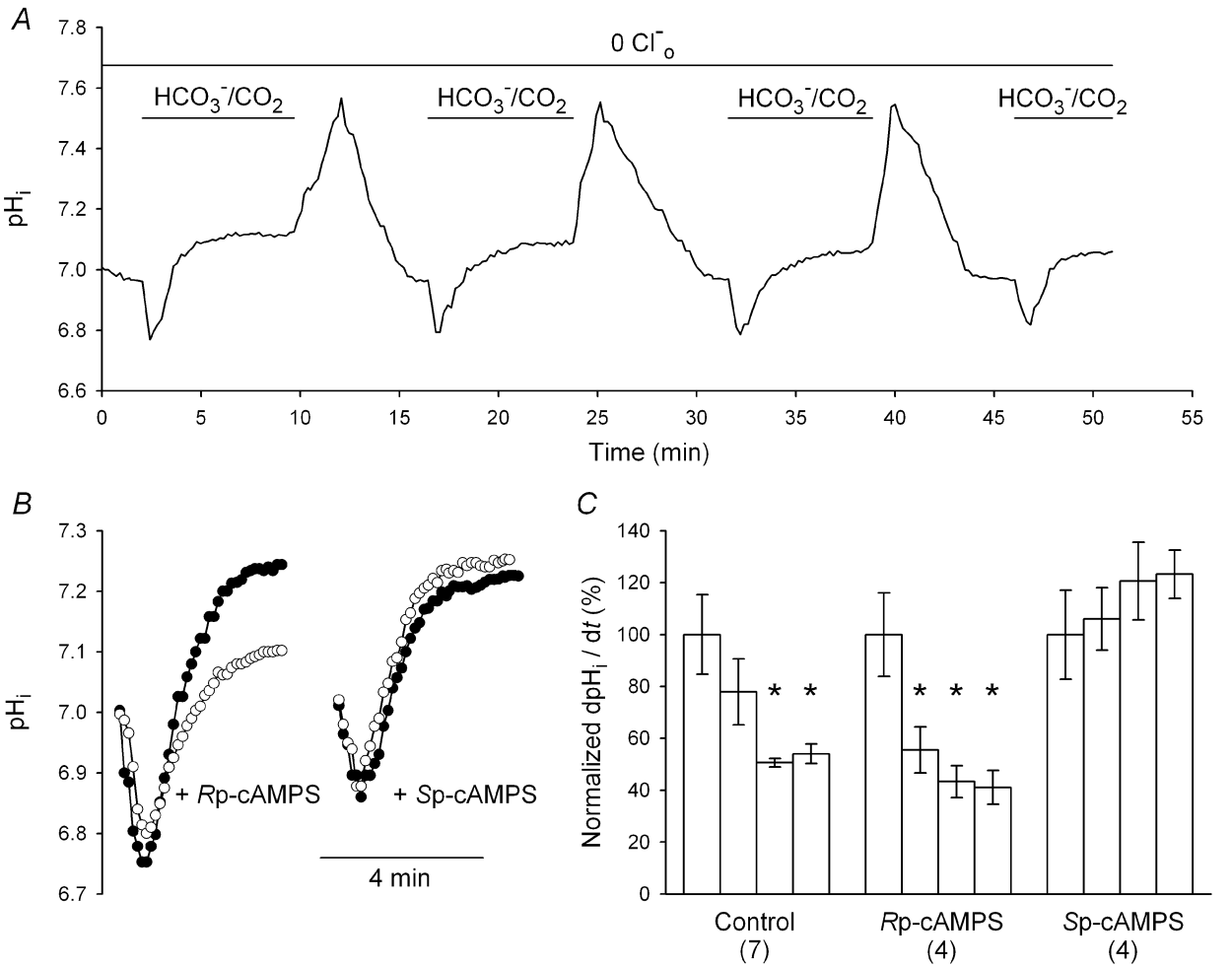

Figure 8. Effects Rp- and Sp-cAMPS on alkalinizations evoked by HCO_3^-/CO_2 under Cl^-_o-free conditions

A, under Cl^-_o-free conditions, repeated exposures to HCO_3^-/CO_2 caused pH_i to transiently decrease and then increase to a new steady-state level; the magnitude of the HCO_3^-/CO_2-evoked increase in pH_i declined with each successive application, resulting in a progressive reduction in the rate at which pH_i reached each new steady-state level (see *C*, Control). *B*, the effects on pH_i of exposing two different low pH_i neurons to HCO_3^-/CO_2 under Cl^-_o-free conditions in the presence of 50 μM Rp-cAMPS (left-hand side) or 25 μM Sp-cAMPS (right-hand side). Under each condition, filled circles identify the record obtained during the first exposure to HCO_3^-/CO_2; traces identified by open circles represent the changes in pH_i observed during the second (Rp-cAMPS) or fourth (Sp-cAMPS) exposures to HCO_3^-/CO_2 in the respective series. *C*, rates of alkalinization observed during consecutive applications of HCO_3^-/CO_2 in the continuous absence of Cl^-_o were calculated at an absolute pH_i value of 7.00 and normalized to the mean rate of alkalinization observed during the first application of HCO_3^-/CO_2 under each experimental condition. The resulting normalized rates of alkalinization are presented as percentage values (± S.E.M.) under control conditions and in the presence of 50 μM Rp-cAMPS and 25 μM Sp-cAMPS. (Number of neurons examined under each experimental condition). * $P < 0.05$ for the difference between the first normalized rate of alkalinization under a given experimental condition.

pH_i neurons upon the addition of HCO_3^- at a constant pH_o (Schwiening & Boron, 1994; Smith *et al.* 1998; also see Baxter & Church, 1996; Bevensee *et al.* 1996). The present study extends these findings to show that, under HCO_3^--containing conditions, the application of DIDS to neurons with initial pH_i values $\leqslant 7.20$ leads to reductions in pH_i. In contrast, in neurons with initial pH_i values > 7.20, the transition from a Hepes- to a HCO_3^-/CO_2-buffered medium caused pH_i to decrease, while pH_i increased following the application of DIDS under HCO_3^--containing conditions. The latter observations, together with the fact that the magnitudes of the HCO_3^-- and DIDS-induced changes in pH_i in neurons with initial pH_i values > 7.20 were greater the more alkaline the initial pH_i, are most easily explained as being due to alterations in Na^+-independent Cl^-–HCO_3^- exchange activity. In many non-neuronal cell types, this transport mechanism is most active at high pH_i values and contributes to pH_i recovery from alkali loads (e.g. Vaughan-Jones, 1986; Olsnes *et al.* 1987; Boyarsky *et al.* 1988; Cassel *et al.* 1988; Ganz *et al.* 1989; Green *et al.* 1990; Kikeri *et al.* 1990; Mugharbil *et al.* 1990; Tønnessen *et al.* 1990; Kramhøft *et al.* 1994; Leem *et al.* 1999). In rat hippocampal neurons, Na^+-independent Cl^-–HCO_3^- exchange has been inferred on the basis of the increase in pH_i observed upon acute reversal of the transmembrane Cl^- gradient (Raley-Susman *et al.* 1993; Baxter & Church, 1996). The present study confirms that pH_i recovery from base loading in CA1 neurons is inhibited by DIDS or the removal of Cl^-_o and can proceed in the absence of Na^+_o.

The fact that the regression fits relating initial pH_i values to the changes in pH_i observed on the addition of HCO_3^- to Hepes-buffered medium (Fig. 1*B*) or the addition of DIDS to HCO_3^--buffered medium (Fig. 1*D*) intersected the respective abscissae at a similar absolute value of pH_i suggests that this value may reflect one towards which CA1 neurons regulate their pH_i when HCO_3^- is available and HCO_3^--dependent pH_i regulating mechanisms are operative. In a manner analogous to Na^+–H^+ exchangers, which possess internal H^+ modifier site(s) that modulate transport activity, the full length isoform of the AE3 anion exchanger, which probably mediates Na^+-independent Cl^-–HCO_3^- exchange in hippocampal neurons (Kopito *et al.* 1989; Raley-Susman *et al.* 1993), may also possess a pH sensor that confers pH_i sensitivity (Lee *et al.* 1991; also see Olsnes *et al.* 1987; Kopito *et al.* 1989; Green *et al.* 1990; Mugharbil *et al.* 1990; Ludt *et al.* 1991; Zhang *et al.* 1996; Stewart *et al.* 2001). Sodium-dependent Cl^-–HCO_3^- exchange activity is also regulated by pH_i (e.g. Boron *et al.* 1979; Boyarsky *et al.* 1988; Kikeri *et al.* 1990; Tønnessen *et al.* 1990); although the identity of the presumed pH sensor remains unknown, histidine-rich regions are present in the cytoplasmic N-terminus of NDCBE1 (which is expressed at the somatic level in rat hippocampal neurons, where it probably mediates Na^+-dependent Cl^-–HCO_3^- exchange; Wang *et al.* 2000; Grichtchenko *et al.* 2001*a*,*b*).

PKA modulation of Na^+-dependent and Na^+-independent Cl^-–HCO_3^- exchange

In rat CA1 neurons under Hepes-buffered conditions, Sp-cAMPS stimulates Na^+–H^+ exchange and thereby increases pH_i, whereas Rp-cAMPS fails to affect either parameter (Smith *et al.* 1998). In contrast, in the present study, both PKA modulators were found to elicit HCO_3^--dependent, DIDS-sensitive changes in pH_i, the directions and magnitudes of which were related to the initial pH_i prior to their application. Although alterations in Na^+-dependent and Na^+-independent Cl^-–HCO_3^- exchange activity (which may themselves be pH_i-dependent; see below) probably contribute to the latter effects, changes in the activities of other, as yet uncharacterized, pH_i regulating mechanisms (see Bevensee *et al.* 1996; Sheldon & Church, 2002) cannot be excluded. Nevertheless, in light of many of the results presented here, these mechanisms are likely to be HCO_3^--dependent and DIDS-sensitive.

The involvement of PKA in the control of Na^+-independent Cl^-–HCO_3^- exchange is suggested by the following observations. First, Rp- and Sp-cAMPS exerted effects on the Cl^-_o-dependent, DIDS-sensitive recovery of pH_i from alkali loads that persisted in the absence of Na^+_o. Second, Rp- and Sp-cAMPS modulated the magnitude of the HCO_3^--dependent, DIDS-sensitive alkalosis seen on acute exposure to Cl^--free medium. With regard to Na^+-dependent Cl^-–HCO_3^- exchange, examination of the effects of PKA modulators on the activity of this transport mechanism in rat CA1 neurons is greatly complicated by the lack of a selective Na^+–H^+ exchange inhibitor. Nevertheless, complementary results were obtained in two distinct experimental protocols, suggesting that PKA does indeed participate in the control of Na^+-dependent Cl^-–HCO_3^- exchange in CA1 neurons. Thus, examined at RT, Sp- and Rp-cAMPS exerted HCO_3^--dependent effects on rates of pH_i recovery from internal acid loads that were attenuated by DIDS. In addition, Rp- and Sp-cAMPS affected the rapidity with which rates of alkalinization in response to repeated applications of HCO_3^-/CO_2 in the absence of Cl^-_o declined.

The control of the activities of neuronal HCO_3^--dependent pH_i regulating mechanisms by intracellular second messengers has not previously been investigated. However, cAMP and/or PKA are known to modify anion exchange activity in a variety of non-neuronal cell types. For example, Na^+-dependent Cl^-–HCO_3^- exchange is stimulated by increases in $[cAMP]_i$ in barnacle muscle fibres (Boron *et al.* 1978) and human bile duct cells (Strazzabosco *et al.* 1997). In contrast, Na^+-independent Cl^-–HCO_3^- exchange may be stimulated, inhibited or unaffected by activation of the cAMP/PKA pathway (e.g. Reuss, 1987; Vigne *et al.* 1988; Green & Kleeman, 1992; Strazzabosco *et al.* 1997; Spirlì *et al.* 1998; Alvarez *et al.* 2001), which emphasizes that the second messenger

control of the activity of a given pH_i regulating mechanism is highly dependent on the cell type in which a given exchanger isoform is expressed. In this regard, both NDCBE1 and AE3 contain consensus sites for phosphorylation by protein kinases, including PKA. (Rat AE3 (GenBank accession number P23348), mouse NCBE (GenBank accession number BAB17922) and human NDCBE1 (GenBank accession number AAC82380) protein sequences were analysed using the ScanProsite sequence analysis tool http://ca.expasy.org/tools/scnpsit1.html). In the present study, Rp- and Sp-cAMPS shifted the pH_i dependence of pH_i recovery from alkali and acid loads and/or changed the slopes of the pH_i *vs.* dpH_i/dt relationships, suggesting that phosphorylation events might contribute to the effects of modulating PKA activity on Na^+-independent and Na^+-dependent Cl^-–HCO_3^- exchange (see Wakabayashi *et al.* 1997). However, we have no evidence to suggest whether the modulation of anion exchanger activities by PKA involves direct phosphorylation of the exchange proteins themselves or of associated regulatory proteins (see Alvarez *et al.* 2001).

Dependence of the PKA modulation of anion exchange activity on initial pH_i

Three intriguing features of the modulation of Cl^-–HCO_3^- exchange by PKA in adult rat CA1 neurons are suggested by the present study (see Table 1). First, in neurons with low ($pH_i \leqslant 7.20$), and possibly high ($pH_i > 7.20$), initial pH_i values, inhibition and activation of PKA lead to opposite effects on the activities of the Na^+-dependent and Na^+-independent anion exchangers. Second, the effects of Rp- and Sp-cAMPS on the activity of a given type of Cl^-–HCO_3^- exchanger (i.e. Na^+-dependent or Na^+-independent) appear to depend on the initial pH_i of a neuron prior to the modulation of PKA activity. Third, the directions of the reciprocal changes in anion exchange activities (inhibition or stimulation) evoked by Sp- and Rp-cAMPS may be opposite in cells with low *vs.* high initial pH_i values. Given the relative lack of experimental data obtained from high pH_i neurons in experiments designed to assess the effects of PKA modulators on the activities of the Na^+-dependent and Na^+-independent Cl^-–HCO_3^- exchangers in relative isolation, these possibilities must remain tentative. Nevertheless, there is some precedence in the literature for the first two of these features. Thus, in guinea-pig ventricular myocytes, not only are the Na^+–HCO_3^- symport and Na^+–H^+ antiport oppositely coupled to α_1-adrenoceptors but also coupling of the two mechanisms to β-receptors is the reverse of that to α_1-receptors (Lagadic-Gossmann & Vaughan-Jones, 1993). And, in Vero cells, non-steroidal anti-inflammatory drugs stimulate Na^+-independent Cl^-–HCO_3^- exchange at $pH_i < 7.0$ whereas, at $pH_i > 7.0$, the antiport is inhibited (cf. the effects of Sp-cAMPS on Na^+-independent anion exchange in the present study); these effects appeared to reflect an action of the drugs to alter the transition from the low to the high activity state of the antiport via drug-induced changes in the activity of an intracellular regulatory system (protein kinase C; Tønnessen *et al.* 1989).

While the mechanism(s) which might underlie the differential pH_i-dependent modulation of Na^+-dependent and Na^+-independent Cl^-–HCO_3^- exchange by PKA are unknown, this feature provides a potential explanation for the observed effects of PKA modulators on steady-state pH_i under HCO_3^--buffered conditions (see Table 1). As noted above, activation of PKA stimulates Na^+–H^+ exchange in both low and high pH_i neurons and thereby evokes a rise in pH_i even in the absence of HCO_3^- (Smith *et al.* 1998). However in the presence of HCO_3^-, the pH_i-dependent effects of Sp-cAMPS on the activities of the Na^+-dependent and Na^+-independent Cl^-–HCO_3^- exchangers could modulate the magnitude of the pH_i increase in a DIDS-sensitive manner, accounting for the experimental results shown in Fig. 2*C* and *D*. Thus, in neurons with initial pH_i values $\leqslant 7.20$, the increase in pH_i elicited by Sp-cAMPS under HCO_3^--buffered conditions is less than that observed in the absence of HCO_3^-, being limited by the concurrent activation of Na^+-independent Cl^-–HCO_3^- exchange and inhibition of Na^+-dependent Cl^-–HCO_3^- exchange. Conversely, in neurons with initial pH_i values > 7.20, a Sp-cAMPS-evoked decrease in Na^+-independent Cl^-–HCO_3^- exchange and increase in Na^+-dependent Cl^-–HCO_3^- exchange could act to augment the rise in pH_i caused by the concomitant activation of Na^+–H^+ exchange. In contrast to Sp-cAMPS, Rp-cAMPS does not affect Na^+–H^+ exchange activity in rat CA1 neurons (Smith *et al.* 1998). However in low pH_i neurons in the presence of HCO_3^-, Rp-cAMPS concurrently stimulates Na^+-dependent Cl^-–HCO_3^- exchange and inhibits Na^+-independent Cl^-–HCO_3^- exchange, resulting in the DIDS-sensitive increase in pH_i observed experimentally (Fig. 2*A* and *B*). Conversely, the DIDS-sensitive decrease in pH_i evoked by Rp-cAMPS in neurons with initial pH_i values > 7.20 (Fig. 2*A* and *B*) could potentially reflect concomitant stimulation of Na^+-independent Cl^-–HCO_3^- exchange and inhibition of Na^+-dependent Cl^-–HCO_3^- exchange.

We can only speculate on the potential functional significance of the present findings, although a number of possibilities exist. For example, rapid increases in $[cAMP]_i$ occur in rat CA1 neurons in slice preparations following anoxia/ischaemia or the application of excitotoxins (e.g. Small *et al.* 1996). Given the low pH_i values typically observed under such conditions, the concurrent effects of PKA activation on Na^+–H^+ exchange and Na^+-dependent and -independent Cl^-–HCO_3^- exchange in low pH_i neurons could contribute to findings that activation of not only Na^+–H^+ exchange but also HCO_3^--dependent, DIDS-sensitive acid loading occurs in hippocampal neurons after

anoxia (Diarra *et al.* 1999; Yao *et al.* 2001; Sheldon & Church, 2002), and that both ischaemia and the application of excitotoxins induce rises in $[Cl^-]_i$ (Inglefield & Schwartz-Bloom, 1998). It is also noteworthy that Cl^-–HCO_3^- exchangers have the potential to regulate the internal concentrations of both anions (Cl^- and HCO_3^-) that are physiologically permeant through $GABA_A$ receptor-operated channels (see Boron *et al.* 1978; Vaughan-Jones, 1986). According to the model presented above, activation of PKA in neurons with pH_i values $\leqslant 7.20$ could cause a positive shift in E_{GABA_A} by stimulating not only Na^+-independent Cl^-–HCO_3^- exchange but also Na^+–H^+ exchange (see Kaila, 1994). This would reduce hyperpolarizing $GABA_A$ responses or even convert them to excitatory ones, with attendant, potentially detrimental, increases in $[Ca^{2+}]_i$ (e.g. Autere *et al.* 1999). Indeed, it has been suggested that Na^+-independent Cl^-–HCO_3^- exchange contributes to the accumulation of Cl^-_i that underlies the excitatory effects of $GABA_A$ receptor activation in hippocampal neurons (Sipilä *et al.* 2000).

Summary

In summary, PKA regulates the activities of the Na^+-dependent and Na^+-independent Cl^-–HCO_3^- exchangers in adult rat CA1 neurons. Inhibition or activation of PKA exerts opposite effects on the activities of the Na^+-dependent and Na^+-independent anion exchangers in neurons with low ($pH_i \leqslant 7.20$) and possibly high ($pH_i > 7.20$) resting pH_i values. Furthermore, the directions of the reciprocal changes in anion exchange activities (inhibition or stimulation) evoked by Sp- and Rp-cAMPS may be opposite in cells with low *vs.* high resting pH_i values, suggesting that pH_i itself may act as a modulator in second messenger-mediated events. Although the precise relationships between the effects of modulating PKA activity on the activities of the transport mechanisms remain to be determined, alterations in Na^+-dependent and Na^+-independent Cl^-–HCO_3^- exchange probably contribute to the effects of modulating PKA activity on steady-state pH_i in CA1 neurons under physiological (i.e. HCO_3^-/CO_2-buffered) conditions, that are themselves dependent on the initial pH_i prior to the modulation of PKA activity. It will be of interest to determine whether additional second messenger systems contribute to the regulation of the activities of Cl^-–HCO_3^- exchangers in hippocampal neurons, as reported in other cell types (e.g. Ludt *et al.* 1991; Green & Kleeman, 1992; Pucéat *et al.* 1998; Alvarez *et al.* 2001).

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