The Regulation of Cytosolic pH in Isolated Presynaptic Nerve Terminals from Rat Brain

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ABSTRACT   Cytosolic pH (pH_i) was measured in presynaptic nerve terminals isolated from rat brain (synaptosomes) using a fluorescent pH indicator, 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF). The synaptosomes were loaded with BCECF by incubation with the membrane-permanent acetoxymethyl ester derivative of BCECF, which is hydrolyzed by intracellular esterases to the parent compound. pH_i was estimated by calibrating the fluorescence signal after permeabilizing the synaptosomal membrane by two different methods. Synaptosomes loaded with 15–90 μM BCECF were estimated to have a pH_i of 6.94 ± 0.02 (mean ± standard error; $n = 54$) if the fluorescence signal was calibrated after permeabilizing with digitonin; a similar value was obtained using synaptosomes loaded with 10 times less BCECF (6.9 ± 0.1; $n = 5$). When the fluorescence signal was calibrated by permeabilizing the synaptosomal membrane to H^+ with gramicidin and nigericin, pH_i was estimated to be 7.19 ± 0.03 ($n = 12$). With the latter method, $pH_i = 6.95 ± 0.09$ ($n = 14$) when the synaptosomes were loaded with 10 times less BCECF. Thus, pH_i in synaptosomes was ~7.0 and could be more precisely monitored using the digitonin calibration method at higher BCECF concentrations. When synaptosomes were incubated in medium containing 20 mM NH_4Cl and then diluted into NH_4Cl-free medium, pH_i immediately acidified to a level of ~6.6. After the acidification, pH_i recovered over a period of a few minutes. The buffering capacity of the synaptosomes was estimated to be ~50 mM/pH unit. Recovery was substantially slowed by incubation in an Na-free medium, by the addition of amiloride ($K_1 = 3$ μM), and by abolition of the Na_o/Na_i gradient. pH_i and its recovery after acidification were not affected by incubation in an HCO_3-containing medium; disulfonic stilbene anion transport inhibitors (SITS and DIDS, 1 mM) and replacement of Cl with methylsulfonate did not affect the rate of recovery of pH_i. It appears that an Na^+/H^+ antiporter is the primary regulator of pH_i in mammalian brain nerve terminals.

INTRODUCTION

Cytosolic pH (pH_i) is a key regulator of many cellular processes (e.g., Roos and Boron, 1981), including the release of neurotransmitters from presynaptic nerve

terminals (Landau and Nachshen, 1975; Cohen and Van der Kloot, 1976; Drapeau and Nachshen, 1988). There is little information, however, about how pH_i is regulated in presynaptic nerve terminals.

Isolated presynaptic nerve terminals (synaptosomes) are useful for studying many aspects of mammalian nerve terminal activity because they retain the functional and morphological properties of intact neuronal tissue (Blaustein et al., 1977; McGraw et al., 1982). The membrane potential is primarily a K^+ diffusion potential (Blaustein and Goldring, 1975). Synaptosomes regulate Ca entry and extrusion (McGraw et al., 1982) and maintain a resting level of cytosolic Ca similar to that found in intact cells (0.1–0.2 μM; Nachshen, 1985). In addition, when synaptosomes are depolarized, there is an increase in Ca entry and in the rate of neurotransmitter release (Blaustein, 1975).

In synaptosomes prepared from rat brain, pH_i is ~7.0 (Richards et al., 1984), well above the equilibrium value expected, given a synaptosomal membrane potential of −60 to −80 mV (pH_i ~ 6.4) (Blaustein and Goldring, 1975; Ramos et al., 1979; Scott and Nicholls, 1980). The synaptosomal pH_i must therefore be regulated by acid-extrusion mechanisms.

At least three types of pH_i-regulatory mechanisms have been described in plasma membranes: (a) a system that requires external Na^+ and HCO_3^- as well as internal Cl^- and is inhibited by disulfonic stilbene anion transport inhibitors; (b) a countertransport of Cl^- for HCO_3^-; (c) a countertransport of extracellular Na^+ and cytosolic H^+ that is inhibited by amiloride. Recently, it has been shown that there is an amiloride-sensitive Na^+/H^+ exchange system in synaptosomes (Sauvaigo et al., 1984). The results of the present study show that it is primarily this system that enables synaptosomes to regulate pH_i and to recover from an acid load.

METHODS

Preparation of Synaptosomes

Synaptosomes were prepared according to the method of Nagy and Delgado-Escueta (1984). In brief, brain samples were homogenized (16 strokes) in ice-cold 0.32 M sucrose with 5 mM HEPES (pH 7.4) and 0.1 mM EDTA. The homogenate was centrifuged at 1,000 g for 10 min. The supernatant was then centrifuged at 12,000 g for 20 min and the supernatant was decanted. The white, fluffy portion of the pellet was resuspended in the medium as above, which contained 7.5% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) and was layered onto a gradient consisting of 10% and 16% Percoll in sucrose medium as above. The gradient was centrifuged at 15,000 g for 20 min. The synaptosomes (banded at the 10%/16% Percoll interface) were removed and diluted with 10 vol of ice-cold Na solution, containing (in millimolar): 145 NaCl, 5 KCl, 3 $MgCl_2$, 1 $CaCl_2$, 10 glucose, 0.005 of the heavy metal chelator diethylenetriamine pentaacetic acid, 0.2 pyruvic acid, 0.2 Na_2PO_4, and 10 HEPES; the pH was adjusted to pH 7.4 with NaOH. The diluted suspension was isolated by centrifugation and the pellet was resuspended in the above solution.

Loading of the Synaptosomes with pH Indicator

The acetoxymethyl ester of 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF-AM), dissolved in dimethylsulfoxide, was added to the resuspended synaptosomes at concentrations of 5–10 μM. The suspension was warmed to 30°C and, after 30 min, diluted 20-

fold with fresh solution. The diluted suspension was centrifuged at 6,000 g for 8 min and the pellet was resuspended and maintained at 30°C.

Synaptosomes that were incubated with BCECF-AM accumulated substantial amounts of the hydrolyzed free acid. After 30 min of incubation in a solution containing 2.5–10 μM BCECF-AM, the synaptosomes accumulated 60–360 pmol BCECF/mg protein (protein content determined by the method of Lowry et al., 1951). If it is assumed that the internal volume of the synaptosomes is ~4 μl/mg protein (Blaustein and Goldring, 1975), the intraterminal concentration of BCECF was 15–90 μM. In some experiments, the BCECF-AM concentration in the loading solution was reduced to 0.2–0.5 μM, resulting in an ~10-fold-lower intraterminal BCECF concentration.

Measurements of BCECF Fluorescence

For measurement of BCECF fluorescence, an aliquot of the synaptosome suspension was diluted 20-fold in resuspension solution and centrifuged for 45 s at 10,000 g in a centrifuge (Microfuge, Beckman Instruments, Inc., Fullerton, CA). The pellet was resuspended in 100 μl and diluted 20-fold in a 1-cm^2 quartz cuvette. BCECF fluorescence was measured with a spectrofluorometer (System 3, Farrand, Valhalla, NY) coupled to a chart recorder. The sample was continuously stirred with a Teflon stir bar and magnetic mixer and the sample chamber was thermostatically maintained at 30°C. Excitation and emission wavelengths of 500 nm (5-nm slit) and 530 nm (10-nm slit) were employed. The emitted light was filtered with a Farrand model 3-70 cutoff filter (50% transmission at 506 nm). In experiments where pH$_i$ was displaced in intact synaptosomes, the rate of recovery of pH$_i$ was estimated from the slope of the initial 15-s period of the recovery.

Determination of BCECF Leakage from the Synaptosomes

BCECF leakage from the synaptosomes was determined by adding aliquots of BCECF-loaded synaptosomes to 20 vol of test solution. At predetermined times, the samples were spun down in a centrifuge. Triton X-100 (0.25%) was added to the pellet and to the supernatant and BCECF fluorescence in both fractions was measured using a spectrofluorometer. The measurement was compared with the fluorescence from a sample of control synaptosomes, treated in a similar manner, to which was added a known amount of BCECF. The extracellular fluorescence was subtracted from the fluorescence of BCECF-loaded synaptosomes in order to estimate pH$_i$.

The loss of accumulated BCECF was initially large (5–10%), which probably reflected the disruption of some synaptosomes during the resuspension procedure. It then proceeded at a slower rate, ~5%/h. Alkalinization of the solution with NaOH, but not with Tris, increased the rate of BCECF efflux appreciably. Acidification of the solution, or resuspension in Na-free solution (Na replaced iso-osmotically by *N*-methylglucamine or choline), had no effect on the rate of BCECF efflux.

Measurement of ^{45}Ca Uptake

^{45}Ca entry was determined as described by Nachshen and Blaustein (1980). Aliquots of the synaptosome suspension were added to equal volumes of solution containing the tracer. ^{45}Ca entry was terminated by rapidly diluting the incubation media with 11 vol of ice-cold Na-free solution (Na replaced by *N*-methylglucamine) containing 1.0 mM EGTA and the diluted suspensions were filtered through glass-fiber filters (2.4 cm diam, GF/A, Whatman, Inc., Clifton, NJ). The filters were washed twice with the ice-cold Na-free solution and placed in vials containing 10 ml scintillation cocktail. The ^{45}Ca content was measured by liquid scintillation spectrometry. The protein content was determined by the method of Lowry et al. (1951).

RESULTS

Determination of pH$_i$

An experiment illustrating how pH$_i$ was determined is shown in Fig. 1A. After a stable fluorescence level was obtained, the synaptosomes were permeabilized by the addition of digitonin (200 μM) so that the fluorescence signal could be calibrated. Small aliquots (0.0005–0.0025 vol) of 1 M Tris (not NaOH; see Methods) or HCl solutions were added to the cuvette. The external pH (pH$_o$) was determined after each addition. Fig. 1B (filled symbols) shows a plot of fluorescence vs. pH$_o$ after the addition of digitonin (corrected for fluorescence of extracellular dye as described in Methods). The arrow indicates the pH at
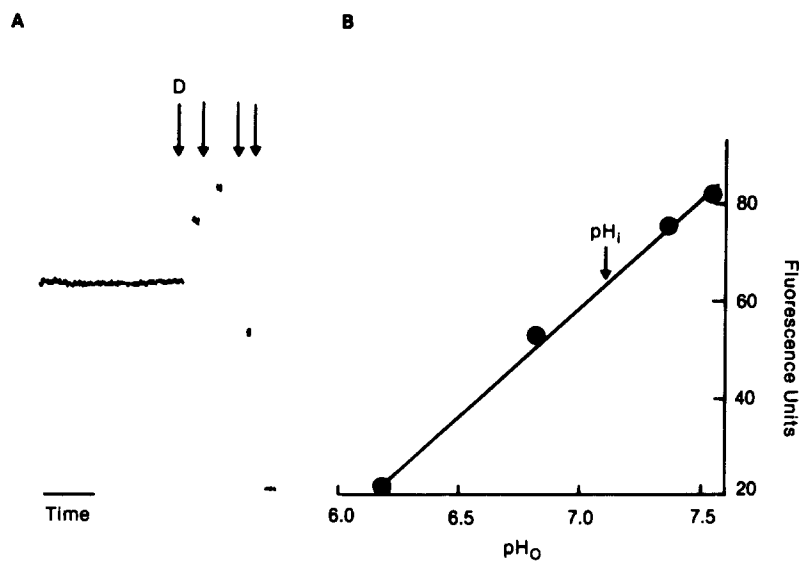

FIGURE 1. Determination of pH$_i$, using digitonin to permeabilize the synaptosomes. (A) Fluorescence was measured in BCECF-loaded synaptosomes for ~5 min. At the first arrow (D), digitonin (100 μM) was added to the synaptosome suspension. At subsequent arrows, small aliquots (0.0005–0.0025 volume) of 1 M Tris or HCl were added to the suspension, changing pH$_o$ from 7.36 initially to 7.54, 6.82, and 6.18, respectively. The time bar corresponds to 2 min. (B) A plot of pH$_o$ vs. fluorescence (corrected for extracellular fluorescence), taken from A. The arrow indicates the fluorescence level obtained with the synaptosome suspension before the addition of digitonin.

which the fluorescence should be equal to that observed with intact synaptosomes before the addition of digitonin. This pH value provided an estimate of pH$_i$ in the synaptosomes of 6.94 ± 0.02 (mean ± standard error; $n = 54$) for synaptosomes loaded with 15–90 μM BCECF. A similar value (pH$_i$ = 6.9 ± 0.1; $n = 5$) was obtained for synaptosomes loaded with 10 times less BCECF.

It has been reported (Rink et al., 1982) that the addition of Triton X-100 or digitonin may result in an underestimation of pH$_i$. An additional calibration method was therefore used. This involved depolarizing the synaptosomes by the

addition of gramicidin and nigericin. Gramicidin makes the synaptosome membranes leaky to cations, so that $[K^+]_i = [K^+]_o$ (Blaustein and Goldring, 1975). Nigericin (0.5 μg/ml) is a K^+/H^+ antiporter (Pressman, 1976). After addition of both agents, pH_i should equal pH_o. It was verified that addition of nigericin and gramicidin did not increase the leakage of BCECF from the synaptosomes above the 5–10% usually found (see Methods); i.e., the dye remained trapped within the synaptosomes.

After addition of gramicidin and nigericin, the pH_o was varied and fluorescence was measured at different pH levels (Fig. 2B, filled symbols; corrected for extracellular fluorescence). The pH corresponding to the fluorescence of synaptosomes before the addition of gramicidin and nigericin was determined
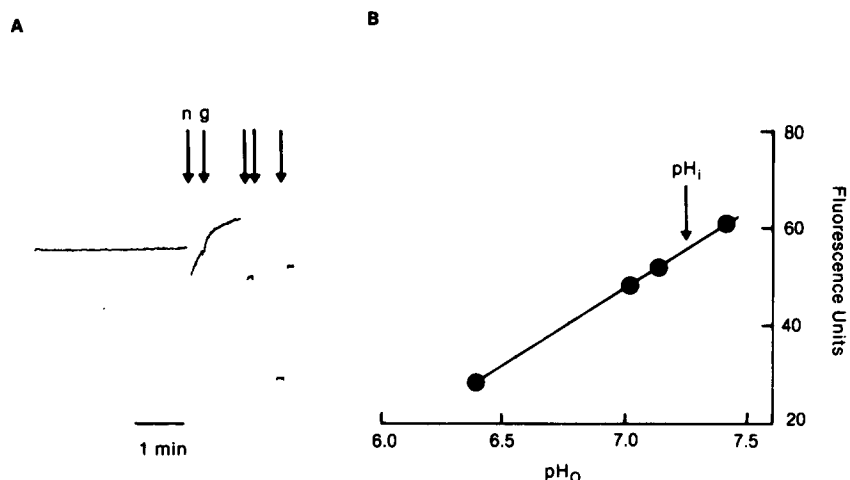

FIGURE 2. Determination of pH_i, using nigericin (0.5 μg/ml) and gramicidin (1 μM) to make pH_o equal pH_i in synaptosomes. (A) Fluorescence was measured in BCECF-loaded synaptosomes for ~5 min. At the first arrow (n), nigericin was added to the suspension; at the second arrow (g), gramicidin was added. Subsequent arrows indicate the addition of Tris or HCl, changing pH_o from 7.42 initially to 7.02, 6.39, and 7.15, respectively. The time bar indicates 1 min. (B) A plot of pH_o vs. fluorescence (corrected for extracellular fluorescence), taken from A. The arrow indicates the fluorescence level obtained with the synaptosome suspension before the addition of nigericin and gramicidin.

(arrow, Fig. 2B). In 12 experiments, pH_i determined in this manner had a value of 7.19 ± 0.03. This estimate of pH_i is shifted upward by 0.25 pH units over the estimate obtained after disruption of the synaptosomal membrane with digitonin. Similar results have been obtained in lymphocytes (Rink et al., 1982) and in proximal tubules (Chaillet et al., 1985).

In another group of 14 experiments, synaptosomes were loaded with 10 times less BCECF and the pH_i estimated by the gramicidin/nigericin method described above was 6.95 ± 0.09 (not shown). Although it is technically more difficult to estimate because of the lower signal-to-noise ratio, the value of pH_i obtained at the lower BCECF concentration is probably more accurate since problems such as compartmentalization of the dye should be reduced. It was concluded that

pH_i in synaptosomes is ~7.0. Calibration by permeabilization with digitonin was an accurate method for monitoring pH_i and, more importantly, signals of greater precision could be obtained at the higher dye concentrations using this method. As would be expected, agents that induced synaptosomal acidification or alkalinization (see Figs. 3 and 4) had no effect on pH_i after the addition of digitonin (data not shown).

Determination of the Buffering Capacity of Synaptosomes

Two different methods were used to estimate the cytosolic buffering capacity of the synaptosomes. The first involved adding NH_4Cl (20 mM) to the synaptosomes. As shown in Fig. 3A, this induced an immediate alkalinization of the
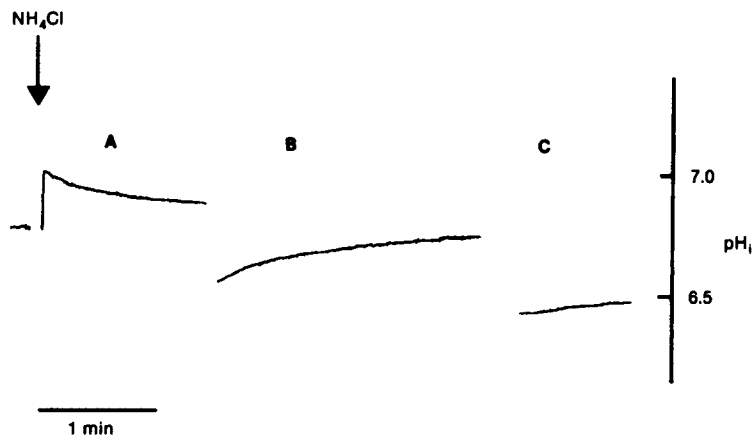

FIGURE 3. NH_4Cl-induced alkalinization and acid rebound. (A) At the arrow, 20 mM NH_4Cl was added to the suspension of synaptosomes. This induced an immediate alkalinization of pH_i. (B) A sample of synaptosomes was resuspended in solution containing 20 mM NH_4Cl. After 3 min, this suspension was mixed with 20 vol of NH_4Cl-free solution in the cuvette of the spectrofluorometer, causing immediate acidification, and fluorescence was measured. The initial rate of recovery of pH_i was 4.0 milli-pH units/s. (C) Same as in B, except that the suspension of synaptosomes was added to Na-free solution in the cuvette. The initial rate of recovery of pH_i was 1.2 milli-pH units/s.

synaptosomes, which presumably reflects the inward diffusion of NH_3 and its subsequent association with H^+ in the cytosol. For each NH_4^+ formed, one intracellular H^+ is consumed; therefore,

$$B = \Delta[NH_4^+]_i/\Delta pH_i, \tag{1}$$

where B is the buffering power, and

$$[NH_4^+]_i = [NH_3]_i \times 10 \exp(pK_a - pH_i). \tag{2}$$

$[NH_3]_i$ is assumed to equal $[NH_3]_o$ at equilibrium and the pK_a for ammonium is taken as 9.2. In 10 experiments, B was estimated to be 53 ± 8 mM/pH unit.

The second method for estimating B involved acid-loading the synaptosomes using CO_2. As shown in Fig. 4A, when synaptosomes were added to a solution

containing 23 mM $NaHCO_3$ (pCO_2, 5%; pH_o 7.4), there was a barely perceptible (0.02 pH unit) acidification of the synaptosomes, produced by an influx of CO_2, which hydrates to yield HCO_3^- and H^+. The largest acidification observed in four similar experiments was 0.1 pH unit. For each HCO_3^- formed, one H^+ is released; therefore,

$$B = \Delta[HCO_3^-]_i/\Delta pH_i \tag{3}$$

and

$$[HCO_3^-]_i = s \cdot pCO_2 \times 10 \exp(pH_i - pK_a), \tag{4}$$

where s is the solubility of CO_2 in cell water (Siesjo, 1962). In four different experiments, B was estimated to be 34 ± 8 mM/pH unit. This value, albeit less accurate, is in reasonable agreement with the estimate found using NH_4Cl, and we therefore conclude that the buffering capacity of the synaptosomes is on the order of 50 mM/pH unit.
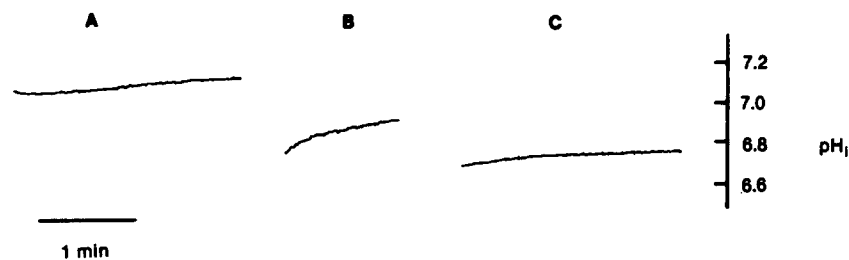

1 min

FIGURE 4. (*A*) Synaptosomes were resuspended, added to 20 vol of solution containing 23 mM $NaHCO_3$, and gassed with 5% CO_2 and fluorescence was immediately measured. (*B*) Synaptosomes were resuspended in solution containing 23 mM $NaHCO_3$ and 5% CO_2, as well as 20 mM NH_4Cl. After 3 min, the suspension was mixed with 20 vol of solution with HCO_3^- and CO_2, without NH_4Cl, to induce acidification. The initial rate of recovery from acidification was 6.4 milli-pH unit/s. (*C*) The synaptosomes were resuspended as in *B*, and mixed with 20 vol of solution containing 23 mM of choline HCO_3 and 5% CO_2. The initial rate of recovery of pH_i was 1.2 milli-pH units/s.

The Effect of pH_o on pH_i

Lowering pH_o diminishes depolarization-induced Ca uptake (Nachshen and Blaustein, 1979) and Ca-dependent, evoked transmitter release (Landau and Nachshen, 1975; Drapeau and Nachshen, 1988) and increases the rate of spontaneous transmitter release (Landau and Nachshen, 1975; Cohen and Van der Kloot, 1975; Drapeau and Nachshen, 1988). It therefore seemed important to determine whether changes in pH_o were accompanied by changes in pH_i. The results in Fig. 5 show that pH_i changed little (from 7.0 to 6.5) as pH_o was lowered from 7.5 to 6.0. The dashed line in Fig. 5 is an "identity" line. The deviation of the data points from this line suggests that pH_i is effectively regulated at both extremes of pH_o.

The effect of pH_i on the rate of acid extrusion is shown in Fig. 6. As in many other types of cells, the rate of acid extrusion is increased by the lowering of pH_i.
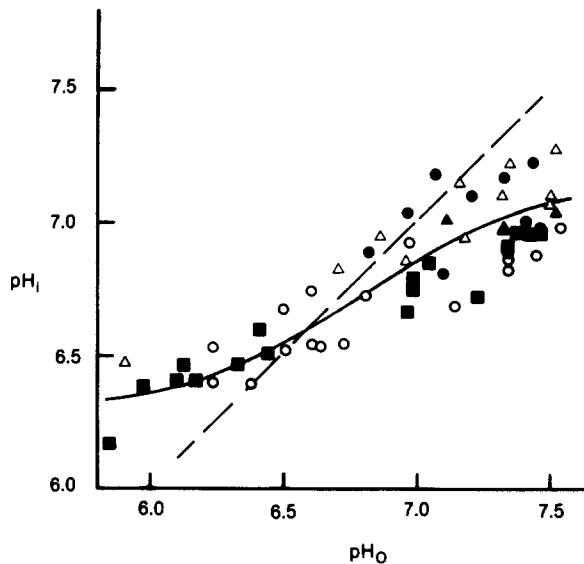
FIGURE 5. The effect of pH_o on pH_i. Results from five experiments are summarized. Synaptosomes were resuspended and pH_o was lowered by the addition of HCl. After the fluorescence signal stabilized (3–4 min), the signal was calibrated with digitonin. The solid line drawn through the points has no theoretical significance. The broken line is a line of identity ($pH_i = pH_o$).

Recovery of Synaptosomes from Acid Load Is Dependent on External Na

When synaptosomes that had been resuspended with a solution containing NH_4Cl (20 mM) were diluted with NH_4Cl-free solution, they immediately acidified (Fig. 3B) and pH_i recovered within a few minutes. pH_i remained low, however, in Na-free solution (Fig. 3C). The rate of recovery of pH_i increased as the concentration of Na in the external solution was increased in the range 0–145 mM (Fig. 7). When synaptosomes were acid-loaded by a 3-min preincubation in Na-free solution containing 20 mM NH_4Cl, the maximum initial rate that was measured in the presence of Na was ~6 milli-pH unit/s. For a cytosolic buffering capacity of ~50 mM/pH unit, this amounted to a proton transfer of ~0.3 mmol/(liter cell water·s).
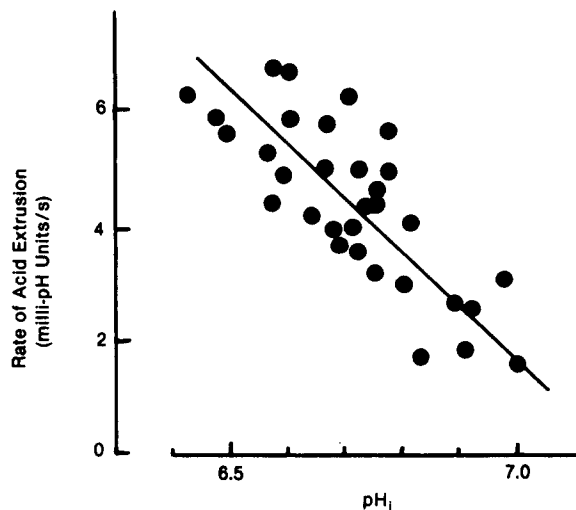

FIGURE 6. The effect of pH_i on the rate of acid extrusion. Synaptosomes were acidified to various levels of pH_i by loading with 5–40 mM NH_4Cl for times ranging from 30 s to 5 min. The NH_4Cl-loaded synaptosomes were then mixed with 20 vol of NH_4Cl-free solution and the initial rate of acid extrusion was determined. Results from six experiments are summarized. The straight line has no theoretical significance.
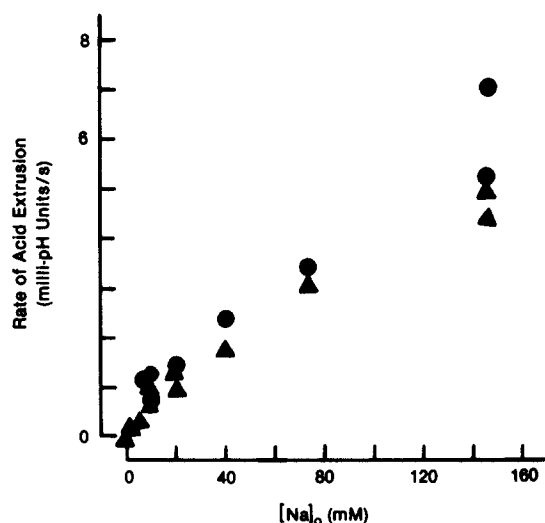
FIGURE 7. The Na^+ dependence of acid extrusion. Synaptosomes were acidified to a level of ~6.5, by a 3-min incubation in solution containing 20 mM NH_4Cl and resuspension in solution without NH_4Cl and with varying amounts of Na^+. Na^+ was replaced isosmotically with n-methylglucamine (circles) or choline (triangles).

The pH_i recovery following an acid load depended not only on external Na, but also on the Na gradient. This was shown by abolishing the Na gradient by including veratridine (100 μM, to increase presynaptic Na conductance) and ouabain (1 mM, to block Na extrusion via the Na pump) in the resuspension solution. After this treatment, the synaptosomes were loaded with Na and the rate of recovery from the acid load decreased (Fig. 8B). This was not a direct effect of ouabain and veratridine on the H-extrusion mechanism since, when the synaptosomes were acid-loaded in Na-free solution, veratridine and ouabain had only a slight effect on the subsequent rate of acid extrusion when the synaptosomes were returned to Na-containing solution (Fig. 8C).

Recovery from Acid Load Is Independent of HCO_3^- and Cl^-

In some cells, pH regulation depends on extracellular HCO_3^- and Cl^-. The effect of these anions on pH_i and on its recovery after an acid load was investigated.
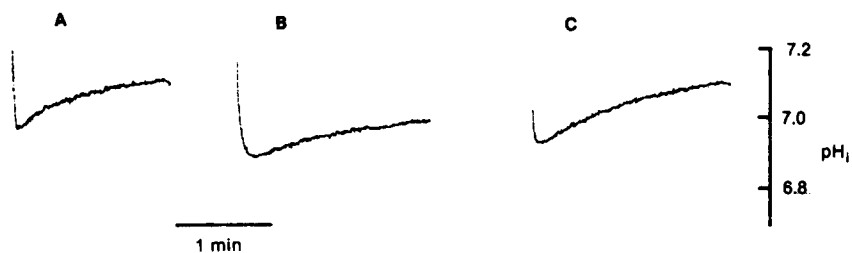

FIGURE 8.   Acid extrusion is dependent on the Na_o/Na_i gradient. (A) Synaptosomes were resuspended in solution with 20 mM NH_4Cl and after 3 min mixed with 20 vol of solution without NH_4Cl. The initial rate of recovery of pH_i was 4.0 milli-pH units/s. (B) Same as in A, except that the resuspension solution also contained 100 μM veratridine and 1 mM ouabain. The initial rate of recovery was 1.3 milli-pH units/s. (C) Synaptosomes were resuspended in Na-free solution with 100 μM veratridine, 1 mM ouabain, and 20 mM NH_4Cl. The suspension was then mixed with 20 vol of Na-containing solution. The initial rate of recovery was 3.6 milli-pH units/s.

pH$_i$ in synaptosomes that were maintained in a solution containing 23 mM HCO$_3^-$ was 6.86 ± 0.05 (n = 9), a value not significantly different from the control value of 6.94 ± 0.02 that was found in medium without HCO$_3^-$. The rate of recovery of pH$_i$ was similar in synaptosomes that had been resuspended in solutions with (Figs. 4B and 9A) or without (Fig. 6) HCO$_3^-$. Also, pH$_i$ recovered only slowly in synaptosomes resuspended in Na-free solution containing 23 mM choline HCO$_3^-$ (Fig. 4C).

The effect of Cl$^-$ on the rate of recovery of pH$_i$ was compared in NH$_4^+$-loaded synaptosomes that were resuspended in solutions containing Na$^+$ and HCO$_3^-$ (46 mM, 10% CO$_2$) made up with either Cl$^-$ or with methanesulfonate as the counteranion. The replacement of Cl$^-$ by methanesulfonate did not decrease the
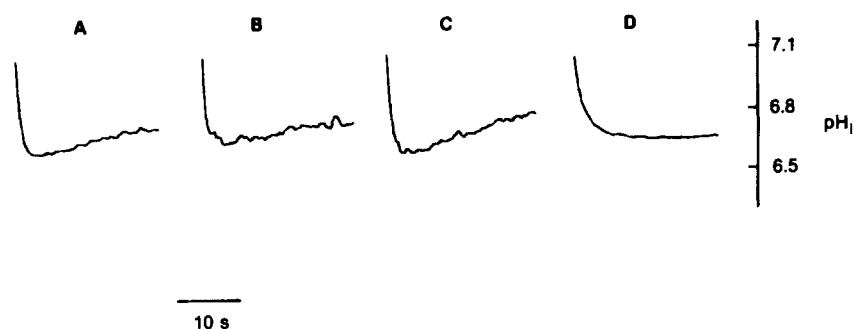


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  A          B          C          D          ┤ 7.1

                                              ┤ 6.8
                                                    pH₁

                                              ┤ 6.5
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10 s

FIGURE 9. Effect of Cl$^-$ replacement, SITS, and amiloride on the recovery of pH$_i$ in HCO$_3^-$-containing medium. (A) Synaptosomes were incubated in medium containing 100 mM NaCl and 46 mM NaHCO$_3$. NH$_4$Cl (20 mM) was added for 3 min. The synaptosomes were then centrifuged and resuspended in HCO$_3^-$-containing medium without NH$_4$Cl in the cuvette. The initial rate of recovery of pH$_i$ was 6.3 milli-pH units/s. (B) Same as in A, except that the cuvette contained a Cl-free solution, consisting of 100 mM Na-methane-sulfonate, 46 mM NaHCO$_3$, 5 mM K-methanesulfonate, and 3 mM MgSO$_4$. All other components (glucose, HEPES, etc.) were as in the control solution. The rate of recovery of pH$_i$ was 5.9 milli-pH units/s. (C) Same as in A, except that the solution in the cuvette also contained 1 mM SITS. The initial rate of recovery of pH$_i$ was 7.1 milli-pH units/s. (D) Same as in A, except that the solution in the cuvette also contained 0.1 mM amiloride. The initial rate of recovery of pH$_i$ was 0.5 milli-pH units/s.

rate of recovery (Fig. 9B). In the same experiment, two inhibitors of Cl$^-$/HCO$_3^-$ countertransport, SITS (Fig. 9C) and DIDS (not shown) (1 mM), were tested and did not decrease the rate of recovery of pH$_i$. These experiments show that an Na-dependent mechanism is the primary process regulating the recovery of pH$_i$ from an acid load in synaptosomes.

Amiloride Inhibits the Recovery of Synaptosomes from Acid Loading

In most systems that have been studied, Na$^+$/H$^+$ exchange is inhibited by micromolar concentrations of amiloride. The effect of 0.1 mM amiloride on the rate of recovery of pH$_i$ in synaptosomes that were resuspended in medium with HCO$_3^-$ (46 mM) is shown in Fig. 9D. There was again a substantial slowing of the recovery of pH$_i$.

The effect of amiloride on the Na-dependent recovery of synaptosomes from

an acid load is shown in Fig. 10 (inset). Recovery was almost completely blocked by 0.1 mM amiloride. The amiloride dose-response curve is also shown in Fig. 10. In solutions containing 72.5 mM Na^+, block was half-maximal with an amiloride concentration of ~3 μM.

Recovery of Synaptosomes from Acid Load Is Accompanied by H^+ Extrusion

The recovery from an acid load could, in principle, be due to internal sequestration of H^+. However, as shown in Fig. 11, the recovery was accompanied by the extrusion of H^+ from synaptosomes. In this experiment, synaptosomes that had
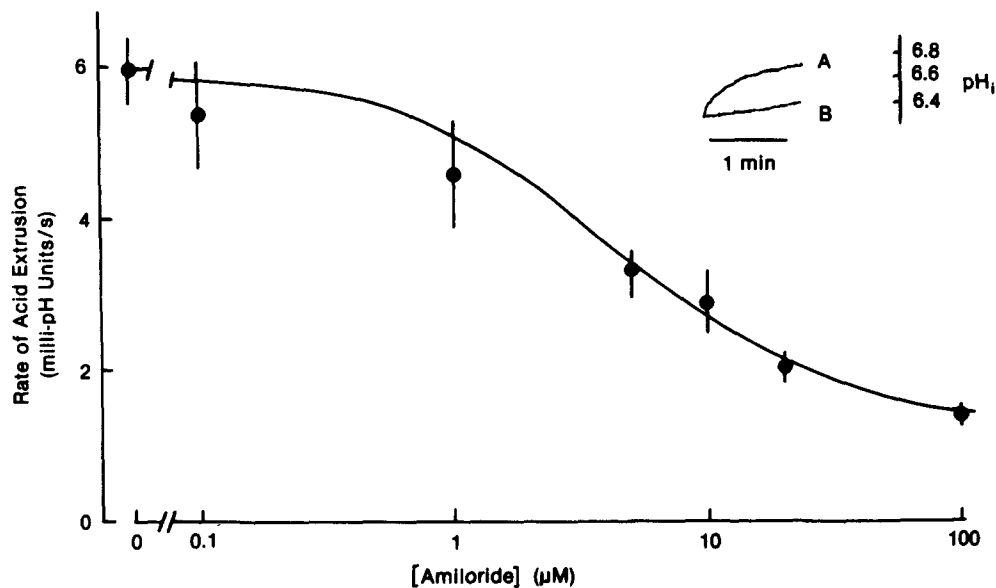

FIGURE 10.   The effect of amiloride on acid extrusion. Synaptosomes were acid-loaded by resuspension in solution with 20 mM NH_4Cl and then mixed with 20 vol of a solution with 72.5 mM Na replaced by choline and varying amounts of amiloride. The initial rate of acid extrusion was then measured. The inset shows the rate of acid extrusion in the absence (A) or the presence (B) of 0.1 mM amiloride. The curve was drawn to the equation $\Delta pH_i = A + \Delta pH_{i(max)}/(1 + [amiloride]/K_1)$, where A is the rate of acid extrusion in the presence of maximal concentrations of amiloride (~1 milli-pH unit/s), $\Delta pH_{i(max)}$ is the increase in the rate of extrusion in the absence of amiloride (5 milli-pH units/s), and K_1 is the apparent amiloride inhibition constant (~3 μM).

not been loaded with BCECF were resuspended in medium that contained a low concentration (0.5 mM) of HEPES buffer and 100 nM BCECF. In Na-containing medium, there was an extracellular acidification that proceeded at a rate of ~0.3 μM/(mg protein·s) (Fig. 11A). When the synaptosomes were acid-loaded by a 3-min preincubation in medium containing 20 mM NH_4Cl and resuspended in NH_4Cl-free medium, the rate of extracellular acidification was greatly increased, to ~1.5 μM/(mg protein·s) (Fig. 11B). If the intrasynaptosomal volume is 4 μl/mg protein (Blaustein, 1975), the proton transfer induced by acid loading is ~0.3 mmol/(liter cell water·s). This value is in accord with the initial rate of

recovery from acid loading measured with BCECF-loaded synaptosomes (see Fig. 3 and related text). In addition, acid extrusion was blocked in Na-free medium (Fig. 11 C) and by 0.1 mM amiloride (Fig. 11 D). Thus, it is evident that recovery of the synaptosomes from acid loading is accompanied by the extrusion of H^+ into the extracellular medium.

Effect of Ca on pH_i and on Recovery from Acid Loading

In some cells, Ca ions can affect pH_i by displacing protons from a common buffer site (Meech and Thomas, 1980; Vaughan-Jones et al., 1983) and by altering the
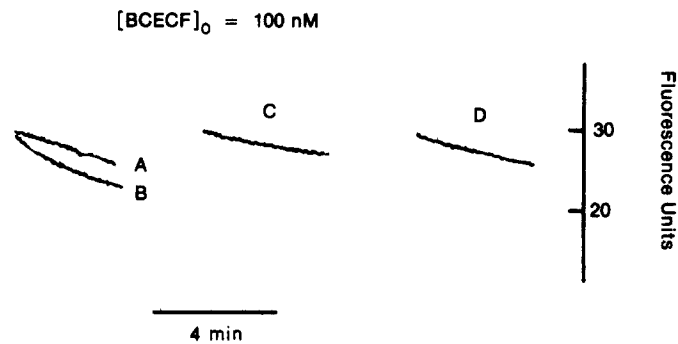

FIGURE 11. Extrusion of H^+ measured with external BCECF. (A) Synaptosomes were resuspended and mixed with 20 vol of lightly buffered medium (0.5 mM HEPES) containing 100 nM of BCECF and fluorescence was measured. The initial rate of acidification of the extracellular solution 59 pmol H^+/s; the rate of acidification of the lightly buffered medium in the absence of synaptosomes was ~20 pmol H^+/s; i.e., the net rate of acid extrusion from the synaptosomes was 39 pmol H^+/s. (B) Synaptosomes were resuspended in solution containing 20 mM NH_4Cl and mixed with 20 vol of lightly buffered solution without ammonia. The initial rate of extracellular acidification was 150 pmol H^+/s (net rate of extrusion = 130 pmol H^+/s). The figure has been offset to the same initial fluorescence level as Fig. 11A, to facilitate a comparison of the slopes. (C) Same as in B, except that the NH_4Cl-loaded synaptosomes were mixed with 20 vol of Na-free solution. The initial rate of extracellular acidification was 30 pmol H^+/s (net rate of extrusion = 10 pmol H^+/s). (D) Same as in B, except that the Na solution contained 0.1 mM amiloride. The initial rate of extracellular acidification was 31 pmol H^+/s (net rate of extrusion = 11 pmol H^+/s).

Na^+ gradient (Deitmer and Ellis, 1980). Since presynaptic nerve terminals undergo significant changes in cytosolic Ca levels during the normal course of their function, the effect of Ca on pH_i and the recovery from acidification was examined.

In one set of experiments, synaptosomes were resuspended in Na-free solution containing either 1 mM Ca or no added Ca and 0.1 mM EGTA. In both batches of synaptosomes, there was a lowering of pH_i. The acidification proceeded at an initial rate of 0.11 milli-pH units/s in the synaptosomes without Ca; at the end of 6 min, pH_i was 6.46. The acidification proceeded at a similar initial rate, 0.09 milli-pH units/s, in the synaptosomes with Ca; at the end of 6 min, pH_i was 6.50. In parallel experiments, it was determined that Ca uptake (measured radioiso-

topically) amounted to ~40 nmol/mg protein. Thus, in contrast to observations in other preparations, Ca loading of nerve terminals was not accompanied by acidification. In another set of experiments, synaptosomes were acid-loaded by incubation for 3 min in Na-free medium containing 20 mM NH_4Cl (see above), either with or without Ca, followed by dilution of the synaptosomes into NH_4Cl-free medium with or without Ca. The recovery rate was similar in both batches: 3.75 milli-pH units/s in the synaptosomes without Ca and 3.2 milli-pH units/s in synaptosomes with Ca.

pH_i in synaptosomes that were suspended in Na-containing solution without Ca and with 0.1 mM EGTA was 6.97 ± 0.01 ($n = 8$), not significantly different from the control value of 6.96 ± 0.01 ($n = 8$) in synaptosomes resuspended in solution with 1 mM Ca.

Veratridine activates presynaptic Na channels, thereby depolarizing the nerve terminals and opening voltage-dependent Ca channels (Blaustein, 1975), and results in a large increase of cytosolic Ca from ~100 nM to >1 μM (Nachshen, 1984). Nonetheless, addition of veratridine (50–100 μM) to a suspension of synaptosomes incubated in Na-containing medium with 1 mM Ca had no effect on pH_i.

In the accompanying article (Drapeau and Nachshen, 1988), it is shown that lowering pH_i (to at least 6.0) has no effect on cytosolic Ca (as measured with the Ca-specific fluorescent dye fura-2). Thus, physiological changes in pH_i or pCa_i in synaptosomes do not appear to affect each other.

DISCUSSION

The purpose of this study was to determine the mechanisms regulating pH_i in nerve terminals. pH_i and the buffering capacity of synaptosomes were estimated as 7.0 and 50 mM/pH unit, respectively, and these resemble estimates obtained with other preparations (Roos and Boron, 1981, Table 13). The Na^+/H^+ antiporter was found to be the primary mechanism for regulating pH_i in rat brain synaptosomes and is remarkably similar to the antiporter found in a variety of mammalian cells, e.g., fibroblasts (Moolenaar et al., 1984), lymphocytes (Grinstein et al., 1984), heart cells (Piwnica-Worms et al., 1985), and collecting tubules (Chaillet et al., 1985). Thus, it is activated by Na^+ and by acidification of the cytosol, and is blocked by micromolar concentrations of amiloride.

In contrast to invertebrate neuronal tissue (squid axon: Russell and Boron, 1982; snail neuron: Thomas, 1982; crayfish stretch receptor: Moser, 1985), where pH regulation is accomplished via an $Na^+/HCO_3^-/Cl^-$ antiporter, we found no evidence for the presence of either an $Na^+/HCO_3^-/Cl^-$ antiporter or a Cl^-/HCO_3^- antiporter in nerve terminals isolated from rat brain. It thus appears that the regulation of pH_i in nerve endings from rat brain is accomplished by a mechanism common to a variety of vertebrate tissues rather than by a neuron-specific process.

Although an Na^+/H^+ antiporter was the only identifiable regulator of pH_i, it was noted in several experiments that even in the absence of external Na^+, or in the presence of amiloride, there was some recovery of pH_i from an acid load (cf. Chaillet et al., 1985). One possibility is that intraterminal organelles (e.g., mitochondria) can sequester protons; another possibility is that they are extruded via an as yet unidentified mechanism at the plasma membrane, e.g., an H^+-

ATPase (Steinmetz and Andersen, 1982). Nonetheless, the conclusion of this study is that the major route for H^+ extrusion from mammalian nerve endings is via exchange for extracellular Na^+.

Ca entry did not affect pH_i and its recovery after an acid load. As shown in the accompanying article (Drapeau and Nachshen, 1988), lowering pH_i had no effect on pCa_i. These results suggest that the regulation of the cytosolic level of either of these ions in nerve terminals is insensitive to the level of the other ion, although both depend on the Na^+ gradient. The mutually insensitive cytosolic H^+- and Ca^{2+}-buffering mechanisms contrast with the regulation of transmitter release shown to depend on both pCa_i and pH_i (Drapeau and Nachshen, 1988).

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