Effects of noradrenaline on intracellular pH in acutely dissociated adult rat hippocampal CA1 neurones

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1. We examined the effects of noradrenaline on steady-state intracellular pH (pH_i) and the recovery of pH_i from internal acid loads imposed by the NH_4^+ prepulse technique in hippocampal CA1 neurones acutely dissociated from adult rats.

2. Under nominally HCO_3^--free conditions, acid extrusion was accomplished by a Na^+-dependent mechanism, probably the amiloride-insensitive variant of the Na^+–H^+ exchanger previously characterized in both fetal and adult rat hippocampal neurones. In the presence of external HCO_3^-, acid extrusion appeared to be supplemented by a Na^+-dependent HCO_3^-–Cl^- exchanger, the activity of which was dependent upon the absolute level of pH_i.

3. Noradrenaline evoked a concentration-dependent and sustained rise in steady-state pH_i and increased rates of pH_i recovery from imposed intracellular acid loads. The effects of noradrenaline were not dependent upon the presence of external HCO_3^- but were blocked by substituting external Na^+ with *N*-methyl-D-glucamine, suggesting that noradrenaline acts to increase steady-state pH_i by increasing the activity of the Na^+–H^+ exchanger.

4. The effects of noradrenaline on steady-state pH_i and on rates of pH_i recovery from imposed acid loads were mimicked by β_1- and β_2-, but not α-, adrenoceptor agonists. The β-adrenoceptor antagonist propranolol blocked the ability of noradrenaline to increase both steady-state pH_i and rates of pH_i recovery from acid loads.

5. The effects of noradrenaline on steady-state pH_i and on pH_i recovery rates following acid loads were not dependent on changes in $[Ca^{2+}]_i$. However, the effects of noradrenaline were blocked by pre-treatment with the adenylate cyclase inhibitor 2′,5′-dideoxyadenosine and the cAMP-dependent protein kinase inhibitors R_p-adenosine-3′,5′-cyclic monophosphorothioate (sodium salt; Rp-cAMPS) and *N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide (H-89).

6. Forskolin, an activator of endogenous adenylate cyclase, and 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, mimicked the ability of noradrenaline to increase both steady-state pH_i and rates of pH_i recovery from imposed acid loads, as did Sp-cAMPS, a selective activator of cAMP-dependent protein kinase. The effect of forskolin on steady-state pH_i was blocked by pre-treatment with Rp-cAMPS whereas the effect of Sp-cAMPS was enhanced by pre-treatment with the protein phosphatase inhibitor, okadaic acid.

7. Noradrenaline also increased steady-state pH_i and rates of pH_i recovery from imposed acid loads in cultured postnatal rat hippocampal neurones. In this preparation, the effects of noradrenaline were occluded by 18–24 h pre-treatment with cholera toxin.

8. We conclude that noradrenaline increases the activity of the Na^+–H^+ exchanger in rat hippocampal neurones, probably by inducing an alkaline shift in the pH_i dependence of the antiport, thereby raising steady-state pH_i. The effects of noradrenaline are mediated by β-adrenoceptors via a pathway which involves the α-subunit of the stimulatory G-protein G_s ($G_{s\alpha}$), adenylate cyclase, cAMP and the subsequent activation of cAMP-dependent protein kinase which, in turn, may phosphorylate the exchange mechanism.

It is now apparent that changes in intracellular pH (pH_i) can both reflect and influence neuronal function. Activation of either γ-aminobutyric acid$_A$ ($GABA_A$) or *N*-methyl-D-aspartate receptors, for example, leads to falls in pH_i attributable, respectively, to a net efflux of HCO_3^- ions across $GABA_A$ receptor-activated anion channels or to a rise in $[Ca^{2+}]_i$ and (possibly) subsequent activation of Ca^{2+}–H^+ exchange (see Kaila, 1994; Trapp *et al.* 1996). Changes in pH_i evoked by amino acid neurotransmitters may, in turn, represent a physiologically important facet of their mechanism of action given that neuronal ionic conductances, the activities of intracellular second messenger systems, and buffering and transport mechanisms for various intracellular ions are all sensitive to changes in pH_i (e.g. Dipolo & Beaugé, 1982; Daumas & Andersen, 1993; Vignes *et al.* 1996; Tombaugh & Somjen, 1997).

Intracellular pH is critically dependent on the activity of pH_i-regulating mechanisms. In peripheral cell types, it is well established that the activities of pH_i-regulating mechanisms, including Na^+–H^+ and HCO_3^-–Cl^- exchangers, are highly controlled (reviewed by Mahnensmith & Aronson, 1985; Grinstein *et al.* 1989; Noël & Pouysségur, 1995; Wakabayashi *et al.* 1997). Depending upon cell type, the activities of these exchangers can be modulated not only by changes in extracellular pH (pH_o) and pH_i but also by a wide variety of external stimuli, including transmitters, mitogens and hormones. Furthermore, it is clear that surface receptors for these and other extracellular agents are coupled to changes in the activities of pH_i-regulating mechanisms through divergent intracellular signalling pathways. The importance of the modulation of pH_i by changes in the activities of pH_i-regulating mechanisms has been highlighted in studies of cell metabolism, growth and proliferation, where pH_i shifts transform the functional state of cells (Busa & Nuccitelli, 1984; Mahnensmith & Aronson, 1985; Grinstein *et al.* 1989; Wang *et al.* 1997). However, in contrast to peripheral cell types, the possibility that external stimuli such as neurotransmitters may change pH_i in mammalian central neurones by specifically altering the activities of pH_i-regulating mechanism(s) has not been explored. In the present study, we have investigated the ability of noradrenaline to modulate the activities of the acid extrusion mechanisms present in hippocampal CA1 neurones acutely dissociated from adult rats. Not only does the hippocampus receive a dense noradrenergic innervation from the locus coeruleus (Loy *et al.* 1980) but also catecholamines, including noradrenaline, are known to affect the activities of pH_i-regulating mechanisms in a variety of peripheral cell types (see Discussion).

Portions of this work have been presented in abstract form (Smith & Church, 1997; Brett & Church, 1998).

METHODS

Cell preparation

Acutely dissociated adult rat hippocampal CA1 neurones. Acutely dissociated adult rat hippocampal CA1 neurones were prepared using a modification of the procedure described by Mody *et al.* (1989). Male Wistar rats (200–240 g) were anaesthetized with 3% halothane in air, decapitated and the brains rapidly removed and placed in ice-cold (4–6 °C) HCO_3^-/CO_2-buffered saline (see below) equilibrated with 5% CO_2–95% O_2. Transverse hippocampal slices (450 μm) were then prepared and allowed to recover for at least 1 h at 32 °C in HCO_3^-/CO_2-buffered saline. To isolate CA1 neurones, three slices were removed from the incubation chamber and enzymatically digested for 30 min at 32 °C in 2 ml of HCO_3^-/CO_2-buffered saline containing 1·5 mg ml^{-1} protease type XIV (Sigma Chemical Co.). The CA1 regions were then removed under a dissecting microscope and triturated with fire-polished Pasteur pipettes in 0·5 ml of Hepes-buffered saline (see below) to which 3 mM $NaHCO_3$ had been added in place of NaCl. The triturated suspension was deposited onto an 18 mm poly-D-lysine-coated glass coverslip mounted in a perfusion chamber so as to form the base of the chamber. Neurones were allowed to adhere to the substrate for 15 min at room temperature (18–22 °C) before being loaded with fluorophore.

Cultured postnatal rat hippocampal neurones. Primary cultures of postnatal hippocampal neurones were prepared from 4- to 5-day-old Wistar rat pups as previously described (Sidky & Baimbridge, 1997). In brief, animals were anaesthetized with CO_2, decapitated and the hippocampi removed. Neurones were plated at a density of 3×10^5 cells cm^{-2} on glass coverslips, treated with 5-fluoro-deoxyuridine to arrest glial cell proliferation and were maintained in a 5% CO_2 atmosphere at 36 °C in serum-free, N_2-supplemented Dulbecco's modified Eagle's medium (Life Technologies, Burlington, ON, Canada) containing 22 mM $NaHCO_3$. Cultured neurones were used 7–14 days after plating in experiments in which prolonged (18–24 h) pre-incubation with cholera toxin was required.

Solutions and test compounds

The standard 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; titrated to pH 7·35 (at 37 °C) with 10 M NaOH. In standard HCO_3^-/CO_2-containing media, Hepes was isosmotically replaced by NaCl and solutions contained 20 mM $NaHCO_3$, by equimolar substitution for NaCl, together with the constituents listed above (pH 7·35 after equilibration with 5% CO_2–95% air). During perfusion with HCO_3^--containing media, the atmosphere in the recording chamber contained 5% CO_2–95% air.

Solutions containing 20 mM NH_4Cl were prepared by equimolar substitution for NaCl. When external Na^+ was omitted, *N*-methyl-D-glucamine ($NMDG^+$) or Li^+ were employed as substitutes in Hepes-buffered media; choline was employed as the substitute in HCO_3^-/CO_2-buffered media. When external Cl^- was omitted, gluconate was substituted. For Ca^{2+}-free media, Ca^{2+} was omitted, $[Mg^{2+}]$ was increased to 3·5 mM and 200 μM EGTA was added. The pH of each solution was re-measured following every experiment.

5-(*N*-ethyl, *N*-isopropyl)-amiloride (EIPA) and 4,4′-diisothio-cyanatostilbene-2,2′-disulphonic acid (DIDS) were prepared as 50 and 100 mM stock solutions, respectively, in dimethylsulphoxide (DMSO) and stored at −80 °C. Forskolin, 2′,5′-dideoxyadenosine,

1′,9′-dideoxyforskolin and 3-isobutyl-1-methylxanthine (IBMX) were dissolved in DMSO to stock concentrations of 50 mM, whilst *N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide (H-89) was dissolved in DMSO to a stock concentration of 10 mM. Noradrenaline and other adrenoceptor agonists and antagonists were dissolved in ultrapure water (Milli-Q UF Plus, Millipore Ltd) and stored as 50 mM stock solutions with 5 mM sodium ethylenediaminetetraacetate (NaEDTA); on the day of an experiment, stock solutions were dissolved in physiological media to the desired test concentration with 0·3 mM ascorbic acid. NaEDTA and ascorbic acid were employed to delay the oxidative degradation of the compounds, especially important at 37 °C (Hughes & Smith, 1978). The Sp- and Rp-isomers of adenosine-3′,5′-cyclic monophosphorothioate (Sp- and Rp-cAMPS, respectively; sodium salts) were dissolved in ultrapure water to 25 or 50 mM stock solutions. Stock solutions of cholera toxin were prepared in ultrapure water at 500 μg ml^{-1}. Control experiments were performed with DMSO, NaEDTA and ascorbic acid at their final working concentrations and none of the agents affected steady-state pH_i or rates of pH_i recovery from imposed intracellular acid loads (data not shown). Perfusion lines were replaced following each experiment.

Compounds were obtained from Research Biochemicals International with the exceptions of cholera toxin, terbutaline and DIDS (Sigma Chemical Co.); 2′,5′-dideoxyadenosine and H-89 (Biomol Research Laboratories Inc., Plymouth Meeting, PA, USA); Rp- and Sp-cAMPS, Na^+ salts (Biolog Life Science Institute, La Jolla, CA, USA); and 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) and fura-2 AM (Molecular Probes Inc.).

Recording techniques

BCECF and fura-2 were employed to estimate pH_i and $[Ca^{2+}]_i$, respectively. In studies where information was required on the effects of an experimental manoeuvre on both $[Ca^{2+}]_i$ and pH_i, measurements of $[Ca^{2+}]_i$ and pH_i were performed separately in parallel experiments conducted on neurones isolated from the same hippocampus. Neurones were loaded with either 5 μM fura-2 AM for 30 min at 37 °C or 2 μM BCECF-AM for 15 min at room temperature and were then superfused at a rate of 2·4 ml min^{-1} for 15 min with the initial experimental solution at 37 °C prior to the start of an experiment. All experiments were performed at 37 °C. pH_i and $[Ca^{2+}]_i$ were measured using the dual-excitation ratio method, employing a digital fluorescence microscopy system (Atto Instruments Inc., Rockville, MD, USA; Carl Zeiss Canada Ltd, Don Mills, ON, Canada). Details of the methods employed have been presented previously (Church *et al.* 1994; Baxter & Church, 1996). In brief, fluorescence emissions measured at 520 or 510 nm from neurones loaded with BCECF or fura-2, respectively, were obtained from one to three neuronal somata simultaneously (up to 21 neurones simultaneously when using cultured cells) and raw intensity data at each excitation wavelength (488 and 452 nm for BCECF; 334 and 380 nm for fura-2) were corrected for background fluorescence prior to calculation of the ratio. Freshly isolated neurones were chosen for study based on the morphological criteria described by Schwiening & Boron (1994), i.e. a smooth, non-granular appearance with a single major process (presumably an apical dendrite) projecting from one pole of the cell which was at least twice the length of the diameter of the cell body, and the presence of two or more smaller processes at the opposite pole. Analysis was restricted to those neurones able to retain BCECF (as judged by raw emission intensity values recorded during excitation at 452 nm) throughout the course of an experiment (see Bevensee *et al.* 1996). In order to reduce photobleaching of the fluorophores and cell damage, the intensity of the 100 W mercury arc lamp was reduced by 50% and neutral density filters were placed in the light path. The one-point high-$[K^+]$/nigericin technique was employed to convert background-corrected BCECF emission intensity ratios (I_{488}/I_{452}) into pH_i values as described (Baxter & Church, 1996). For the thirteen full calibration experiments utilized in analysing all data, the values of pK_a (−log of the dissociation constant of the fluorophore), $R_{n(min)}$ (the minimum obtainable value for the normalized ratio) and $R_{n(max)}$ (the maximum obtainable value for the normalized ratio) were (mean ± S.E.M.) 7·17 ± 0·03, 0·45 ± 0·03 and 2·03 ± 0·04, respectively. Values for $R_{n(min)}$ and $R_{n(max)}$ were derived from non-linear least-squares regression fits to background-subtracted ratio *versus* pH data which, in turn, were obtained in full calibration experiments (see Baxter & Church, 1996). Periodically, brief fluctuations in the incident radiation from the arc lamp produced variations in emission intensities. In order to smooth the graphical representation of the pH_i *versus* time records, a moving average (period = 3) was applied to all records shown (see Baxter & Church, 1996). Calibration of the fura-2 signal was not attempted and the effects of experimental manoeuvres on $[Ca^{2+}]_i$ are presented as changes in background-corrected I_{334}/I_{380} ratio values.

Experimental procedures and data analysis

The effects of changes in perfusate composition and pharmacological treatments were examined on both steady-state pH_i and on rates of pH_i recovery from internal acid loads imposed by the NH_4^+ prepulse technique. In each experiment in which rates of pH_i recovery were examined, two consecutive intracellular acid loads were imposed, the first being employed to calculate control rates of pH_i recovery for a given neurone and the second being performed under the influence of a pharmacological or other treatment. The mean percentage difference between rates of pH_i recovery following two consecutive acid loads imposed in the absence of a test treatment was established in control experiments. In twenty-one neurones, the difference observed in the overall rate of pH_i recovery between the second and first acid loads was a 3 ± 22% (mean ± S.D.) increase. Therefore, in any given experiment, rates of pH_i recovery under the influence of a test treatment were considered to be different from control rates of pH_i recovery only if they displayed a mean overall increase greater than 47% or a mean overall decrease greater than 41% (i.e. a mean ± 2 S.D. difference from the overall control rate of pH_i recovery established in the same cell). Only neurones which exceeded these criteria were considered to have responded to a test treatment and only data from these neurones underwent further analysis.

Control rates of pH_i recovery were compared with rates of pH_i recovery under a test condition at the same absolute values of pH_i. 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 the test treatment was determined. The mean of the resultant percentage differences was then calculated and employed to describe the overall effect of the test treatment on the rate of pH_i recovery. In addition, a formal statistical comparison was made between rates of pH_i recovery (evaluated at 0·05 pH_i unit increments from the point of maximum acidification) under control and test conditions. For a given absolute value of pH_i, control rates of pH_i recovery and rates of pH_i recovery under a given test condition were grouped separately and

Student's paired two-tailed t test was employed to assess statistical significance. Net acid efflux in nominally HCO_3^--free media was calculated as the product of the measured rate of recovery of pH_i (dpH_i/dt) from an imposed acid load at a given pH_i value and the intrinsic intracellular buffering power (β_i) at the same pH_i value. Values for β_i were calculated from the equation:

$$\beta_i = 94{\cdot}45 - (pH_i \times 11{\cdot}28),$$

which was derived by Bevensee *et al.* (1996) in acutely dissociated rat hippocampal CA1 neurones.

Results are reported as means ± S.E.M. with the accompanying n value referring either to the number of neurones from which data were obtained (acutely dissociated cells) or, in cultured neurones, to the number of cell populations (i.e. number of coverslips) examined. Statistical comparisons were performed using Student's two-tailed t test, paired or unpaired as appropriate, with a 95% confidence limit.

RESULTS

Characterization of acid extrusion mechanisms

Acid extrusion from adult rat hippocampal CA1 neurones is reported to be governed by an amiloride-insensitive Na^+–H^+ exchanger and a Na^+-dependent HCO_3^-–Cl^- exchanger (Schwiening & Boron, 1994; Bevensee *et al.* 1996). Initially, we performed a limited series of experiments to assess whether these mechanisms contribute to acid extrusion in our preparation of acutely dissociated adult rat hippocampal CA1 neurones.

In nominally HCO_3^--free medium, resting pH_i was $7{\cdot}29 \pm 0{\cdot}01$ ($n = 439$). The values of resting pH_i had a broad range (pH 6·6–7·9) and, in agreement with Bevensee *et al.* (1996), their distribution was best fitted with the sum of two Gaussian distributions with means at $pH_i = 6{\cdot}91 \pm 0{\cdot}01$ and $7{\cdot}43 \pm 0{\cdot}01$ (Fig. 1*A*). Application of the amiloride analogue EIPA (50 μM), a pharmacological inhibitor of Na^+–H^+ exchange in a wide variety of cell types, did not alter resting pH_i ($n = 3$) and had no effect on rates of pH_i recovery from imposed intracellular acid loads ($n = 4$; data not shown). However, the removal of external Na^+ (substitution with $NMDG^+$) evoked a rapid $0{\cdot}30 \pm 0{\cdot}03$ pH unit fall in pH_i ($n = 15$; Fig. 2*A*) and blocked the recovery of pH_i from intracellular acid loads ($n = 5$; Fig. 2*B*), suggesting that a Na^+-dependent acid extrusion mechanism contributes to the maintenance of steady-state pH_i under HCO_3^--free conditions. As shown in Fig. 2*C*, the replacement of external Na^+ with Li^+ was marked by an initial acidification but pH_i recovered in the continued absence of Na^+ ($n = 4$; also see Baxter & Church, 1996). In addition, when Na^+-free, Li^+-substituted medium was applied at the point of maximum acidification following an NH_4^+ prepulse, pH_i recovery still occurred ($n = 6$; Fig. 2*D*). The results indicate that acid extrusion under nominally HCO_3^--free conditions is governed by a Na^+-dependent mechanism that can also transport Li^+ in exchange for internal protons. This mechanism is likely to be the amiloride-insensitive variant of the Na^+–H^+ exchanger previously characterized in detail by Schwiening & Boron (1994) and Bevensee *et al.* (1996).

In HCO_3^-/CO_2-buffered saline, resting pH_i was $7{\cdot}20 \pm 0{\cdot}03$ ($n = 55$). As was the case under HCO_3^--free conditions, values of resting pH_i under HCO_3^--containing conditions had a broad range (pH 6·6–7·5) although the distribution of steady-state pH_i values in the presence of HCO_3^- was unimodal and best fitted by a negatively skewed asymmetric logistic function with a modal value of pH_i 7·28 (Fig. 1*B*). Initially, we explored the effect on steady-state pH_i of the transition from a HCO_3^-/CO_2-free medium at pH 7·35 to a medium buffered with HCO_3^-/CO_2 at the same pH_o. Upon

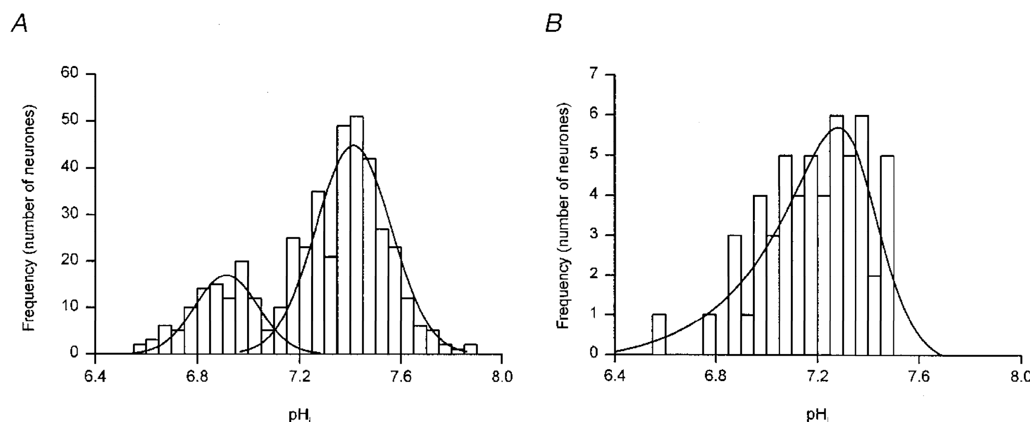

Figure 1. Distribution of steady-state pH_i

A, a frequency histogram (bin width = 0·05 pH units) of steady-state pH_i values for 439 acutely dissociated adult rat hippocampal CA1 neurones during perfusion with pH 7·35 Hepes-buffered medium. The distribution was fitted best with the sum of two Gaussian distributions with means at $pH_i = 6{\cdot}91 \pm 0{\cdot}01$ and $7{\cdot}43 \pm 0{\cdot}01$. *B*, a frequency histogram (bin width = 0·05 pH units) of steady-state pH_i values for 55 acutely dissociated adult rat hippocampal CA1 neurones during perfusion with pH 7·35 HCO_3^-/CO_2-buffered medium. The distribution was negatively skewed and was fitted best with an asymmetric logistic function with a modal value at $pH_i = 7{\cdot}28$.

exposure to HCO_3^-/CO_2-buffered medium, an increase in pH_i typically occurred, the magnitude of which was dependent upon the initial resting level of pH_i in Hepes-buffered medium (Fig. 3*A*). The results, which are in agreement with those of Schwiening & Boron (1994) and Bevensee *et al.* (1996) in the same cell type, indicate that HCO_3^--dependent mechanism(s) can contribute to the maintenance of steady-state pH_i in adult rat hippocampal CA1 neurones. This possibility was further suggested by the fact that, in six additional neurones with an initial resting $pH_i < 7{\cdot}3$ in Hepes-buffered medium, the increase in pH_i expected upon exposure to HCO_3^-/CO_2-buffered medium was blocked by 300 μM DIDS (Fig. 3*B*); applied in the nominal absence of HCO_3^-/CO_2, 200–300 μM DIDS failed to affect steady-state pH_i ($n = 8$; data not shown). In neurones with resting $pH_i < 7{\cdot}3$, replacing external Cl^- with gluconate under HCO_3^-/CO_2-buffered conditions evoked a pH_i increase of $0{\cdot}15 \pm 0{\cdot}02$ pH units ($n = 8$; Fig. 3*C*). In turn, the 0 $[Cl^-]_o$-induced alkalinization was blocked by 300 μM DIDS ($n = 5$; Fig. 3*C*), consistent with its mediation by a carrier coupling HCO_3^- and Cl^- fluxes. Finally, removal of Na^+ from the perfusion medium in the presence of HCO_3^- caused a $0{\cdot}35 \pm 0{\cdot}10$ pH unit fall in pH_i ($n = 3$), similar to the change observed under HCO_3^--free conditions, and blocked pH_i recovery from imposed intracellular acid loads ($n = 3$; data not shown).

The above findings are entirely consistent with the literature concerning acid extrusion in both cultured fetal (Raley-Susman *et al.* 1991; Baxter & Church, 1996) and acutely dissociated adult CA1 (Schwiening & Boron, 1994; Bevensee *et al.* 1996) rat hippocampal neurones. They indicate that acid extrusion in our preparation of adult rat CA1 neurones under HCO_3^--free conditions is mediated by an amiloride-insensitive Na^+–H^+ exchanger. Under HCO_3^--containing conditions, acid extrusion appears to be supplemented by the activity of a DIDS-sensitive, Na^+-dependent HCO_3^-–Cl^- exchanger, the activity of which is dependent upon the absolute level of pH_i.

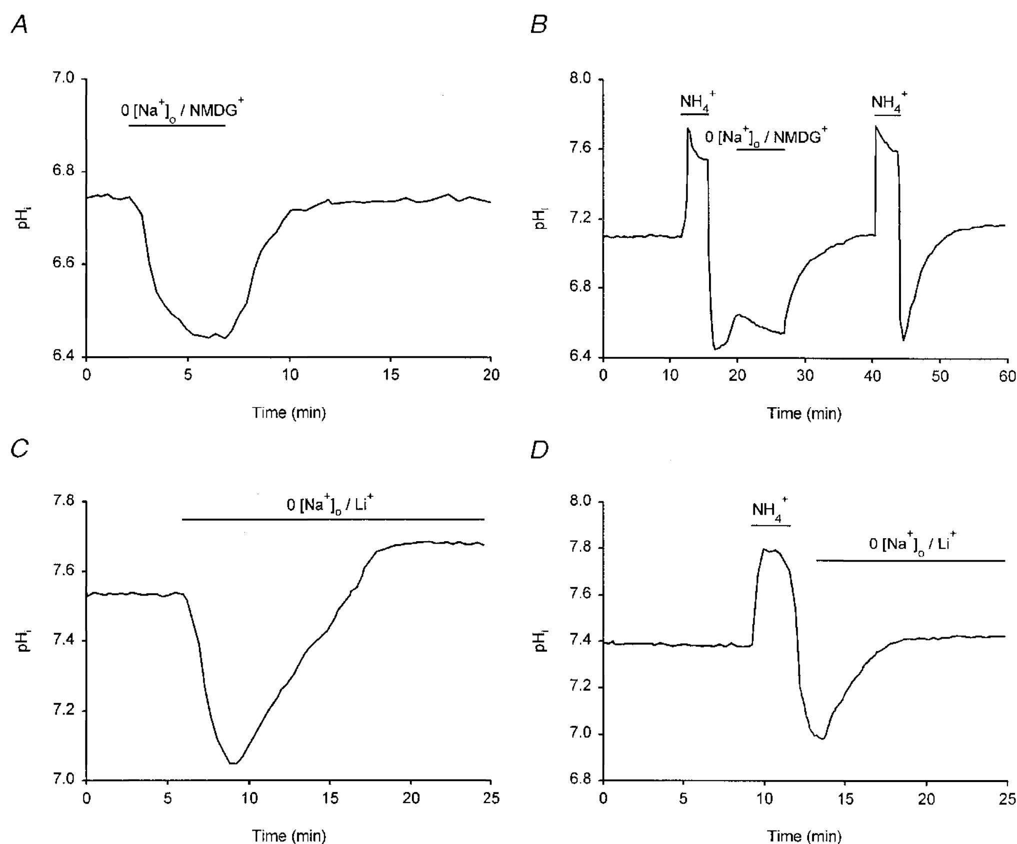

Figure 2. Steady-state pH_i and acid extrusion in the absence of HCO_3^-

A, the removal of external Na^+ ($NMDG^+$ substitution) for the period indicated by the bar above the trace caused pH_i to fall by ~0·3 pH units. pH_i recovered when external Na^+ was reintroduced. *B*, following the first NH_4^+-induced internal acid load, the removal of external Na^+ (replacement with $NMDG^+$) reversibly interrupted pH_i recovery. A second acid load was then performed and pH_i recovery was allowed to take place in the continued presence of external Na^+. *C*, the replacement of external Na^+ with Li^+ evoked a transient intracellular acidification followed by a recovery of pH_i despite the continued absence of external Na^+. *D*, pH_i recovery from an imposed intracellular acid load could take place in the absence of external Na^+ when Li^+ was employed as the substitute cation. Each record was obtained from a different neurone under HCO_3^-/CO_2-free, Hepes-buffered conditions at a pH_o of 7·35.

Effects of noradrenaline on pH_i

Steady-state pH_i. Under HCO_3^-/CO_2-buffered conditions, application of 10 μM noradrenaline evoked, after a 3–5 min delay, an increase in steady-state pH_i of $0{\cdot}19 \pm 0{\cdot}02$ pH units in 6/7 neurones tested (in the remaining neurone, noradrenaline had no effect). A similar rise in steady-state pH_i of $0{\cdot}21 \pm 0{\cdot}02$ pH units was observed under nominally HCO_3^--free conditions in 18/20 neurones tested (Fig. 4*A*; $P > 0{\cdot}1$ for difference to change in pH_i evoked in the presence of HCO_3^-; noradrenaline was without effect in the remaining two neurones). The increase in pH_i evoked by noradrenaline under Hepes-buffered conditions was concentration dependent; 5 and 20 μM noradrenaline increased steady-state pH_i by $0{\cdot}11 \pm 0{\cdot}01$ ($n = 4$) and $0{\cdot}24 \pm 0{\cdot}04$ ($n = 3$) pH units, respectively. Under both HCO_3^--free and HCO_3^--containing conditions, the increase in pH_i persisted following the washout of noradrenaline (see Fig. 4*A*) and for as long as stable recordings could be maintained (up to 60 min). Beneath the pH_i trace in Fig. 4*A* are shown the background-subtracted I_{452} values which were employed in the measurement of pH_i; the stability of the I_{452} values indicates that the persistent nature of the rise in

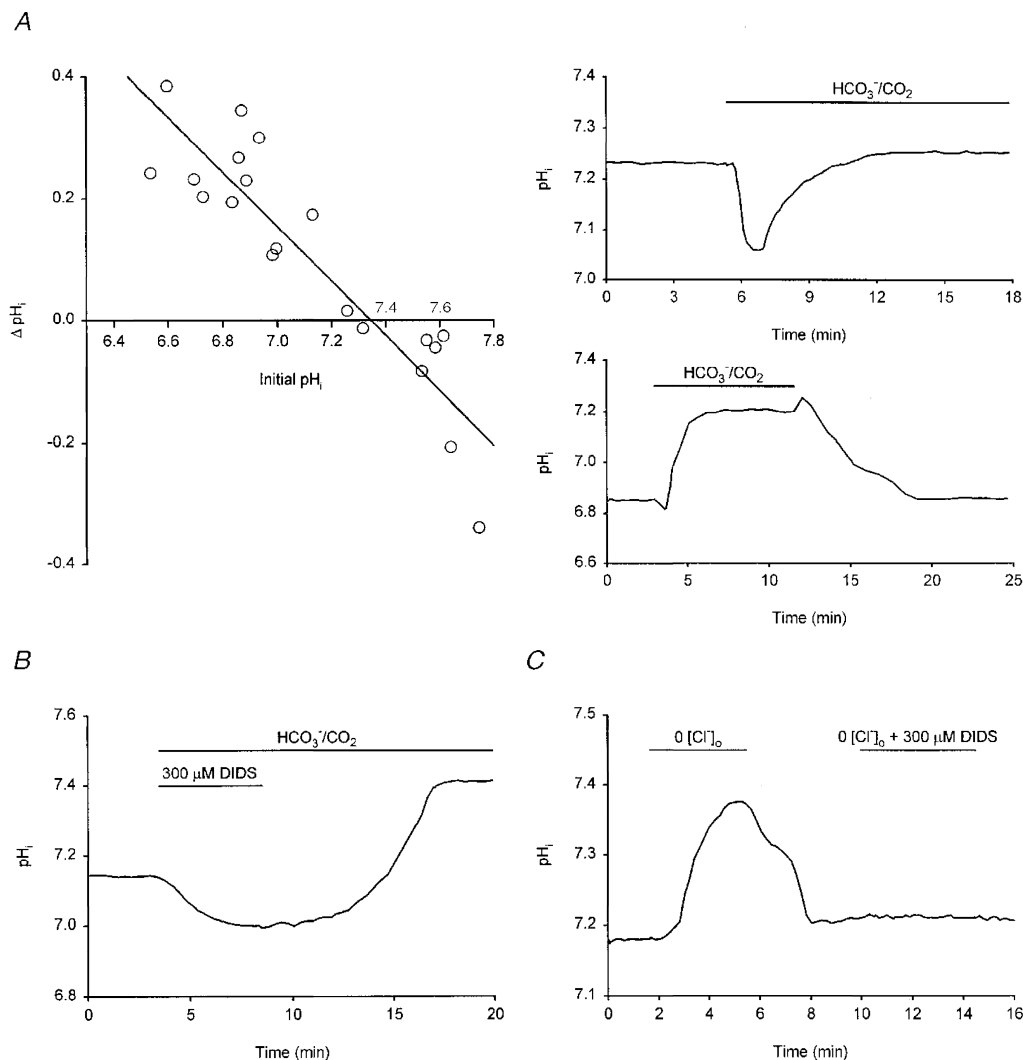

Figure 3. Steady-state pH_i in the presence of HCO_3^-

A, to the left, the change in steady-state pH_i evoked by the addition of HCO_3^- at a constant pH_o (7·35) is plotted against initial pH_i in the absence of HCO_3^- ($n = 20$). The line shown is a linear least-squares best fit to the data points indicated. To the right are records showing the effect of introducing HCO_3^- on pH_i in two different neurones with initial pH_i values in Hepes-buffered medium of ~7·25 (upper record) and ~6·85 (lower record). In both cases, pH_o was 7·35 throughout. *B*, in a different neurone with an initial pH_i in Hepes-buffered medium of ~7·1, the introduction of a HCO_3^-/CO_2-buffered medium containing 300 μM DIDS did not evoke an increase in pH_i. An intracellular alkalinization of ~0·25 pH units above the resting level occurred upon the removal of DIDS from the HCO_3^-/CO_2-buffered medium. Extracellular pH was 7·35 throughout. *C*, in a fourth neurone, the removal of external Cl^- in the presence of HCO_3^- evoked an ~0·2 pH unit internal alkalinization which was abolished by the co-application of 300 μM DIDS.

steady-state pH_i evoked by noradrenaline was not an artifact produced by a decline in I_{452} values consequent upon a deterioration of membrane integrity (see Methods). Because the increase in steady-state pH_i evoked by noradrenaline was similar in the presence or absence of HCO_3^-, it is unlikely to reflect primarily changes in the activities of HCO_3^--dependent pH_i-regulating mechanisms. However, application of 10 μM noradrenaline under HCO_3^-- and Na^+-free ($NMDG^+$-substituted) conditions failed to affect steady-state pH_i in 8/8 neurones tested (Fig. 4*B*), consistent with the possibility that the increase in steady-state pH_i might be mediated by an increase in the activity of the Na^+–H^+ exchanger. Note that, in experiments of the type illustrated in Fig. 4*B*, the weak base trimethylamine was employed to elevate pH_i towards normal resting levels in the continued absence of external Na^+.

pH_i recovery from internal acid loads. Noradrenaline (10 μM) increased the rate of pH_i recovery from an imposed acid load in 5/5 neurones tested in the presence of HCO_3^- and in 15/17 neurones tested under HCO_3^--free conditions; overall rates of pH_i recovery were increased by 193 ± 28% ($n = 5$) and 161 ± 20% ($n = 15$; Fig. 4*C*), respectively ($P > 0.1$). In the remaining two neurones tested under

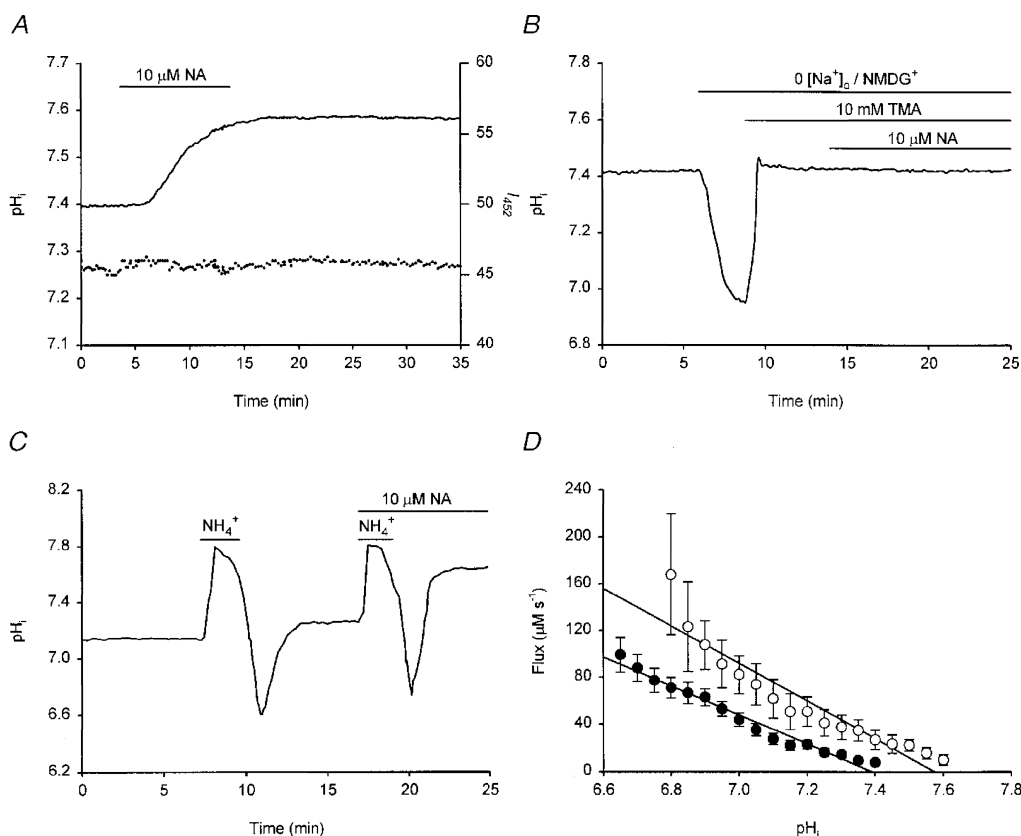

Figure 4. The effect of noradrenaline on steady-state pH_i and the recovery of pH_i from imposed intracellular acid loads

All traces were obtained under HCO_3^-/CO_2-free, Hepes-buffered conditions. Records shown in *A*–*C* were obtained from different neurones. *A*, a 10 min exposure to 10 μM noradrenaline (NA) evoked, after a short delay, an increase in pH_i (continuous line) which persisted following washout of the catecholamine. The background-subtracted I_{452} values which were employed in the measurement of pH_i are shown beneath the pH_i trace (see Results). *B*, the Na^+ dependence of the noradrenaline-evoked intracellular alkalinization was examined by substituting $NMDG^+$ for external Na^+. This produced a fall in pH_i which was returned towards the normal resting value by the addition of 10 mM trimethylamine (TMA). Under these conditions, noradrenaline failed to elicit an increase in steady-state pH_i. *C*, following the first NH_4^+-induced intracellular acid load, pH_i was allowed to recover. A second acid load was then performed in the presence of 10 μM noradrenaline, which increased the rate of pH_i recovery from the imposed internal acidification. pH_i recovered to a higher steady-state level in the presence than in the absence of noradrenaline. *D*, the pH_i dependence of net acid extrusion in the presence (○) and absence (●) of 10 μM noradrenaline. Continuous lines represent the least-squares linear regression fits to the data points indicated for each experimental condition. Data points were obtained from 15 experiments of the type illustrated in *C*; where missing, standard error bars lie within the symbol areas. Noradrenaline significantly ($P < 0.05$) increased H^+ efflux at each absolute value of pH_i and shifted the pH_i dependence of acid extrusion to the right.

HCO_3^--free conditions, 10 μM noradrenaline evoked a 21% decrease and an 11% increase in the overall rate of pH_i recovery. In fifteen paired experiments of the type shown in Fig. 4*C*, which were conducted under Hepes-buffered conditions, pH_i recoveries from imposed acid loads under the influence of 10 μM noradrenaline were compared with control pH_i recoveries at the same absolute values of pH_i. The resulting plots of the pH_i dependence of net acid efflux before and after noradrenaline application are presented in Fig. 4*D*. Noradrenaline (10 μM) significantly increased H^+ efflux ($P < 0.05$ at all absolute values of pH_i) and shifted the pH_i dependence of acid extrusion by ~0·2 pH units in the alkaline direction, suggesting that noradrenaline activates Na^+–H^+ exchange by changing the cytoplasmic pH sensitivity of the antiport (see Grinstein *et al.* 1989; Wakabayashi *et al.* 1997). As was the case for the effect of noradrenaline on steady-state pH_i (see above), the effect of noradrenaline to increase rates of pH_i recovery from internal acid loads required the presence of external Na^+. Thus, in five neurones examined under HCO_3^-/CO_2-free conditions in which the recovery of pH_i from an imposed acid load took place in the presence of 10 μM noradrenaline (see Fig. 4*C*), removal of external Na^+ ($NMDG^+$ substitution) blocked pH_i recovery in a manner identical to that observed in the absence of noradrenaline (see Fig. 2*B*).

As the noradrenaline-evoked increase in steady-state pH_i was abolished under external Na^+-free conditions (Fig. 4*B*), it is unlikely to reflect alterations in intrinsic intracellular buffering power (β_i). In addition, as an indication of the apparent intracellular buffering power, we quantified the increase in pH_i caused by exposure to 20 mM NH_4Cl by taking the difference between the steady-state pH_i immediately prior to the application of NH_4^+ and the maximum pH_i immediately after its application. Under nominally HCO_3^--free conditions, application of NH_4^+ evoked a $0·51 \pm 0·04$ pH unit rise under both control conditions and in the presence of 10 μM noradrenaline ($n = 17$ in each case). As the alkaline shift evoked by NH_4^+ was similar in the presence or absence of noradrenaline, changes in β_i are unlikely to underlie the marked increases in acid extrusion rates evoked by the neurotransmitter (also see Bevensee *et al.* 1996). In this regard, it has previously been found that changes in pH_i evoked by adrenoceptor agonists in a variety of peripheral cell types do not reflect changes in β_i or background acid loading rates (e.g. Guo *et al.* 1992; Lagadic-Gossmann & Vaughan-Jones, 1993).

In summary, the results indicate that the rise in steady-state pH_i evoked by noradrenaline in adult rat hippocampal CA1 neurones is mediated by an increase in the activity of the Na^+–H^+ exchanger. Consequently, subsequent experiments were performed under nominally HCO_3^--free, Hepes-buffered conditions.

Pharmacology of the pH_i response to noradrenaline

The adrenergic receptor subtypes mediating the effects of noradrenaline on steady-state pH_i and on acid extrusion following intracellular acid loads were determined by employing receptor subtype-selective agonists and antagonists. β-Adrenoceptor agonists (isoprenaline, dobutamine and terbutaline) were applied in the presence of the full α-adrenoceptor antagonist, phentolamine (1–10 μM), whereas media containing the α-adrenoceptor agonist 6-fluoro-noradrenaline also contained the non-selective β-adrenoceptor antagonist, propranolol (10 μM).

The full α-adrenoceptor agonist, 6-fluoro-noradrenaline (10 μM), had no effect on steady-state pH_i in five neurones tested (data not shown) and failed to alter the pH_i dependence of acid extrusion following imposed acid loads in 9/9 neurones examined (Fig. 5*A* and *B*). In contrast, the full β-adrenoceptor agonist isoprenaline (10 μM) mimicked the effects of noradrenaline, increasing both resting pH_i by $0·15 \pm 0·03$ pH units in 6/7 neurones tested and overall rates of pH_i recovery from imposed acid loads by 145 ± 12% in 14/16 neurones examined. Steady-state pH_i in the remaining neurone was not affected by 10 μM isoprenaline whereas in the two remaining cells in which pH_i recovery rates were examined, isoprenaline increased the overall rates by 5 and 31%. In fourteen paired experiments of the type shown in Fig. 5*C*, 10 μM isoprenaline significantly increased acid extrusion following imposed acid loads at all absolute levels of pH_i and shifted the pH_i dependence of acid extrusion by ~0·15 pH units in the alkaline direction (Fig. 5*D*).

The selective β_1- and β_2-adrenoceptor agonists dobutamine and terbutaline, respectively, also mimicked the effects of noradrenaline on pH_i. Experiments with dobutamine were performed in the presence of 10 μM ICI 118,551, a highly selective β_2-adrenoceptor antagonist, as well as phentolamine to further ensure the selectivity of the compound for β_1-adrenoceptors. Dobutamine (1 μM) increased steady-state pH_i by $0·18 \pm 0·03$ pH units in 6/7 neurones examined (Fig. 6*A*; dobutamine had no effect on the remaining neurone) and increased the overall rate of pH_i recovery from internal acid loads by 134 ± 10% in 6/7 neurones tested (Fig. 6*B*; in the remaining neurone, the overall rate of pH_i recovery was increased by 16%). Similarly, terbutaline (1 μM, tested in the presence of phentolamine) increased steady-state pH_i by $0·29 \pm 0·06$ pH units ($n = 5/5$; Fig. 6*C*) and, in 10/10 neurones, increased the overall rate of pH_i recovery from imposed acid loads by 165 ± 11% (Fig. 6*D*). The effect of terbutaline to increase steady-state pH_i was concentration dependent; 0·5 and 10 μM terbutaline increased steady-state pH_i by $0·11 \pm 0·03$ ($n = 3$) and $0·52 \pm 0·04$ pH units ($n = 5$), respectively (Fig. 6*C*). Both dobutamine (1 μM) and terbutaline (1 μM) shifted the pH_i dependence of acid extrusion (derived from experiments of the types shown in Fig. 6*B* and *D*, respectively) to the right by ~0·2 pH units (not illustrated).

The results indicate that the effects of noradrenaline on steady-state pH_i and on acid extrusion following imposed acid loads are mediated by β-adrenoceptors. Consistent with this possibility, application of 10 μM noradrenaline in

the presence of the full β-adrenoceptor antagonist propranolol (20 μM) did not evoke a rise in steady-state pH_i in 4/4 neurones tested (Fig. 6*E*) and failed to affect rates of pH_i recovery from acid loads in 7/7 neurones examined (Fig. 6*F*). Concomitantly, propranolol abolished the noradrenaline-evoked alkaline shift in the pH_i dependence of acid extrusion (not illustrated).

It has been suggested that the bimodal distribution of resting pH_i values observed in adult rat hippocampal CA1 neurones in the absence of HCO_3^- (see Fig. 1*A*) may reflect different functional states of the Na^+–H^+ exchanger (Bevensee *et al.* 1996). We therefore examined whether exposure to noradrenaline or β-adrenoceptor agonists affected the distribution of steady-state pH_i values. As illustrated in Fig. 7*A*, the distribution of steady-state pH_i values for fifty neurones prior to exposure to noradrenaline or a β-adrenoceptor agonist was fitted best by the sum of two Gaussian distributions, with means at pH_i 6·93 ± 0·03 and 7·44 ± 0·02. Upon exposure to noradrenaline, isoprenaline, dobutamine or terbutaline, pH_i alkalinized to a new steady-state level and the distribution of pH_i values was now fitted best by a single Gaussian distribution with a mean at pH_i = 7·54 ± 0·03 (Fig. 7*B*).

Intracellular mechanisms mediating the effects of β-adrenoceptor stimulation on pH_i

In some systems, Na^+–H^+ exchange activity and steady-state pH_i can be modulated by changes in $[Ca^{2+}]_i$ (see Mahnensmith & Aronson, 1985; Wakabayashi *et al.* 1997). In addition, β-adrenoceptor activation or manoeuvres which act to increase the intracellular concentration of adenosine-3′,5′-cyclic monophosphate ($[cAMP]_i$) can enhance Ca^{2+} currents in some cell types (e.g. Chetkovich *et al.* 1991). To

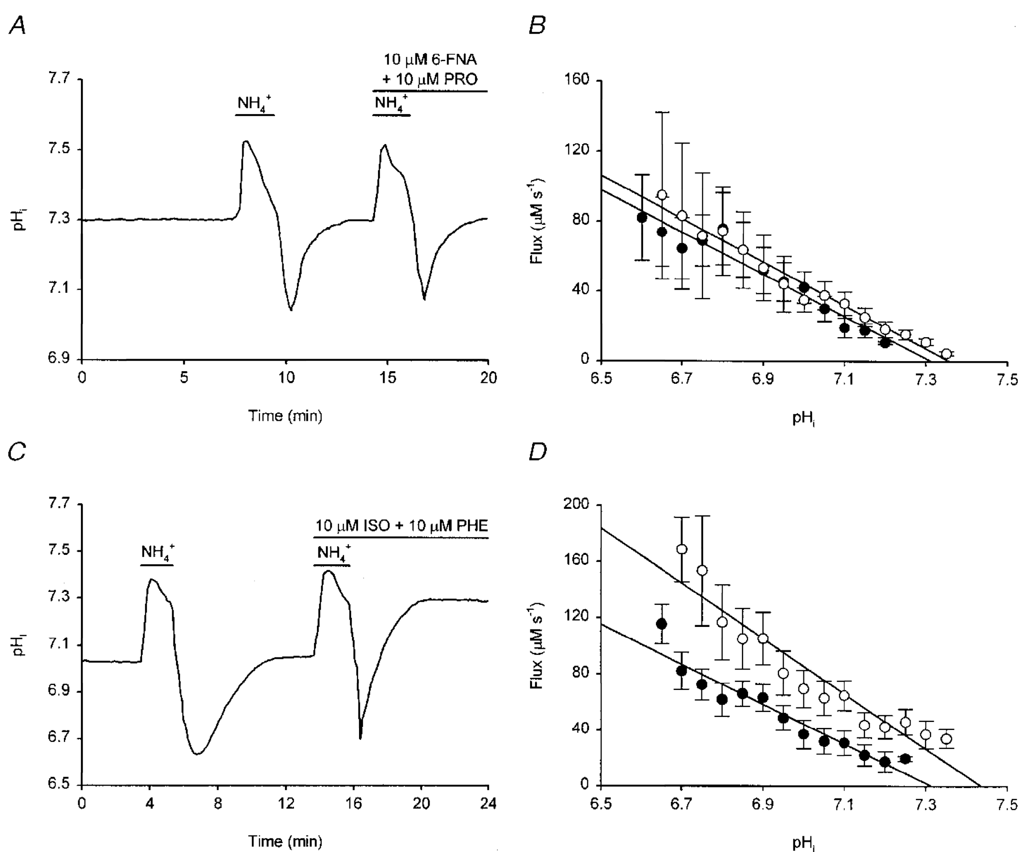

Figure 5. Pharmacology of the pH_i response to noradrenaline

Following an initial acid load and recovery of pH_i to resting levels, a second acid load was performed in the presence of the full α-adrenoceptor agonist 6-fluoro-noradrenaline (6-FNA, 10 μM; applied in the presence of 10 μM propranolol, PRO) (*A*) and in the presence of the full β-adrenoceptor agonist isoprenaline (ISO, 10 μM; applied in the presence of 10 μM phentolamine, PHE) (*C*). In contrast to 6-FNA, isoprenaline increased the rate of pH_i recovery from the intracellular acid load and pH_i recovered to a higher steady-state level than that prevailing under control conditions. To the right are shown the pH_i dependencies of net acid extrusion in the presence (○) and absence (●) of 10 μM 6-FNA (*B*) and 10 μM isoprenaline (*D*). Data were obtained from 9 (in *B*) and 14 (in *D*) paired experiments of the types shown in *A* and *C*, respectively; error bars represent S.E.M. Continuous lines represent the least-squares linear regression fits to the data points indicated for each experimental condition. Acid extrusion in the presence of 6-FNA was not significantly different from control ($P > 0·1$ at each absolute value of pH_i). In contrast, isoprenaline significantly increased acid extrusion ($P < 0·05$ at each absolute value of pH_i).

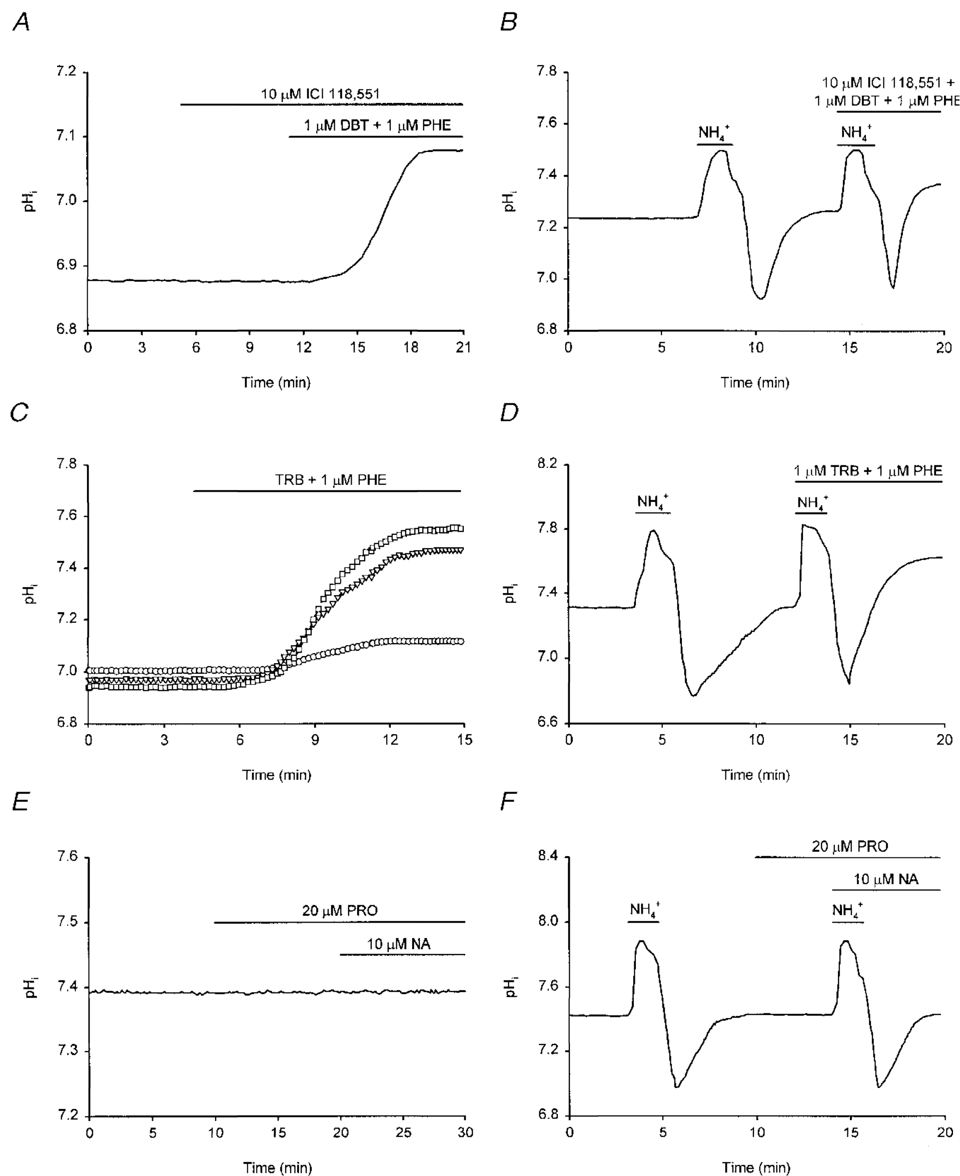

Figure 6. The pH_i response to noradrenaline is mimicked by β_1- and β_2-adrenoceptor agonists

A, exposure to 1 μM dobutamine (DBT), a selective β_1-adrenoceptor agonist, increased resting pH_i. Dobutamine was applied with 1 μM phentolamine (PHE) in the presence of 10 μM ICI 118,551 (a selective β_2-adrenoceptor antagonist). *B*, the neurone underwent an initial acid load and, following the recovery of pH_i, a second acid load was performed in the presence of 1 μM dobutamine, 1 μM phentolamine and 10 μM ICI 118,551. The rate of pH_i recovery was increased in the presence of dobutamine. *C*, representative records from three different neurones, each with a similar resting pH_i, are shown to illustrate the concentration dependence of the effect of the selective β_2-adrenoceptor agonist, terbutaline (TRB), on steady-state pH_i. Terbutaline was applied at 0·5 (○), 1 (▽) and 10 μM (□), in the presence of 1 μM phentolamine in each case. *D*, terbutaline (1 μM; applied in the presence of 1 μM phentolamine) increased the rate of pH_i recovery from an imposed acid load. *E*, applied in the presence of the β-adrenoceptor antagonist propranolol (PRO, 20 μM), 10 μM noradrenaline (NA) failed to increase resting pH_i. *F*, an initial intracellular acid load was imposed under control conditions. Following the recovery of pH_i to resting values, 20 μM propranolol was applied and a second acid load was then performed in the combined presence of 10 μM noradrenaline and 20 μM propranolol. Under these conditions, noradrenaline failed to increase the rate of pH_i recovery from the imposed acid load (compare with Fig. 4*C*). Each record shown in *A–F* was obtained from a different neurone.

investigate whether changes in $[Ca^{2+}]_i$ might contribute to the effects of β-adrenoceptor activation on pH_i in hippocampal neurones, the effects of noradrenaline and isoprenaline on steady-state pH_i and on rates of pH_i recovery following imposed acid loads were examined under external Ca^{2+}-free conditions. Exposure to Ca^{2+}-free medium caused an increase in steady-state pH_i of $0{\cdot}16 \pm 0{\cdot}04$ pH units ($n = 19$; see Fig. 8*A* and *B*). Once pH_i had stabilized at the new resting level, 10 μM noradrenaline evoked a further increase in pH_i of $0{\cdot}18 \pm 0{\cdot}03$ pH units in 12/15 cells tested (Fig. 8*A*; three cells showed no response) whereas 10 μM isoprenaline (tested in the presence of 10 μM phentolamine) evoked an increase in pH_i of $0{\cdot}18 \pm 0{\cdot}02$ pH units ($n = 4/4$; not illustrated). Noradrenaline (10 μM) and isoprenaline (10 μM) also increased overall rates of pH_i recovery from acid loads imposed under Ca^{2+}-free conditions by 145 ± 9% ($n = 6/7$; Fig. 8*B*; in the remaining neurone, the overall rate of pH_i recovery was increased by 8%) and 160 ± 12% ($n = 6/6$), respectively. The increases in steady-state pH_i and rates of pH_i recovery evoked by noradrenaline and isoprenaline in the absence of external Ca^{2+} were not statistically different from the corresponding changes observed in the presence of the cation. Thus, the effects of β-adrenoceptor activation on pH_i are not dependent upon Ca^{2+} influx. In addition, isoprenaline 10 μM had no effect on $[Ca^{2+}]_i$ when applied in the absence of external Ca^{2+} ($n = 5/5$; Fig. 8*C*). We therefore conclude that β-adrenoceptor agonists act in a Ca^{2+}-independent manner to increase acid extrusion and thereby increase steady-state pH_i.

The occupation of β-adrenoceptors by noradrenaline is linked classically to the activation of adenylate cyclase and a subsequent increase in $[cAMP]_i$. We therefore examined whether the effects of noradrenaline on steady-state pH_i and on rates of pH_i recovery from imposed acid loads could be mimicked and attenuated, respectively, by activators and inhibitors of adenylate cyclase. Applied alone, 25 μM forskolin mimicked the actions of noradrenaline, producing a sustained increase in steady-state pH_i of $0{\cdot}23 \pm 0{\cdot}03$ pH units in 16/17 neurones examined (Fig. 9*A*) and increasing the overall rate of pH_i recovery from acid loads by 169 ± 18% ($n = 5/5$; Fig. 9*B*). The steady-state pH_i of the remaining neurone was unaffected by forskolin. Exposure to 25 μM forskolin under external Ca^{2+}-free conditions also evoked a sustained increase in steady-state pH_i of $0{\cdot}20 \pm 0{\cdot}06$ pH units ($n = 4/4$; not illustrated), a result not significantly different from that obtained in the presence of external Ca^{2+}. An inactive analogue of forskolin, 1′,9′-dideoxyforskolin (25 μM), failed to influence steady-state pH_i ($n = 5/5$) or rates of pH_i recovery from imposed intracellular acid loads ($n = 7/7$). The effects of forskolin on steady-state pH_i and on rates of pH_i recovery from imposed acid loads were mimicked by the phosphodiesterase inhibitor IBMX which, like forskolin, acts to raise $[cAMP]_i$. Applied at 200 μM, IBMX evoked an increase in steady-state pH_i of $0{\cdot}20 \pm 0{\cdot}03$ pH units ($n = 12/12$; Fig. 9*C*) and increased the overall rate of pH_i recovery by 142 ± 13% ($n = 14/15$; Fig. 9*D*; the remaining neurone was not affected). In a manner analogous to noradrenaline and β-adrenoceptor agonists, both forskolin (25 μM; Fig. 9*E*) and IBMX (200 μM; not illustrated) shifted the pH_i dependence of acid extrusion in the alkaline direction. Conversely, the effects of noradrenaline on both steady-state pH_i and rates of pH_i recovery from imposed acid loads were attenuated by pre-treatment with the adenylate cyclase inhibitor 2′,5′-dideoxyadenosine. Applied alone, 100 μM

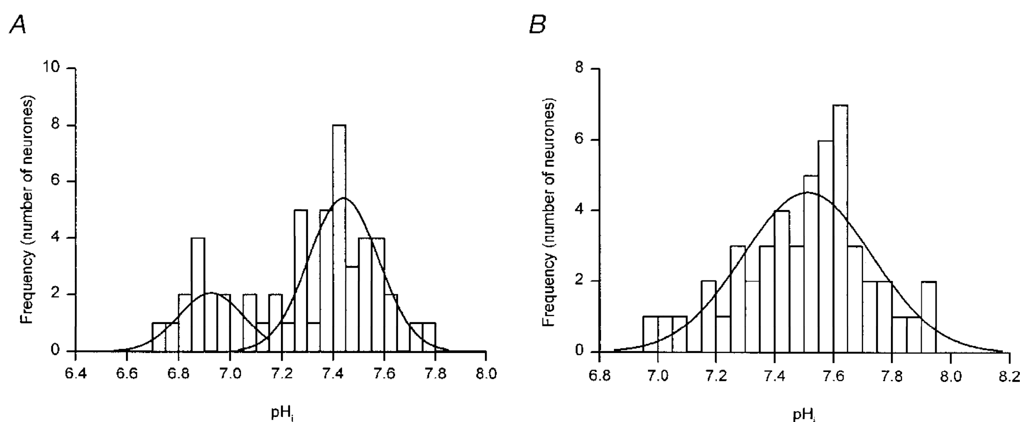

Figure 7. Effect of β-adrenoceptor activation on the distribution of steady-state pH_i values

Distributions of steady-state pH_i from 50 neurones prior to (*A*) and following (*B*) the addition of β-adrenoceptor agonists under HCO_3^-/CO_2-free, Hepes-buffered conditions. *A*, under control conditions the frequency distribution (bin width = 0·05 pH units) was fitted best with the sum of two Gaussian distributions with means at pH_i values of $6{\cdot}93 \pm 0{\cdot}03$ and $7{\cdot}44 \pm 0{\cdot}02$ (compare with Fig. 1*A*). *B*, steady-state pH_i values measured following the addition of noradrenaline, isoprenaline, dobutamine or terbutaline were pooled and the frequency distribution was fitted best with a single Gaussian distribution with a mean at a pH_i of $7{\cdot}54 \pm 0{\cdot}03$.

2′,5′-dideoxyadenosine did not markedly affect steady-state pH_i ($n = 9/9$). However, in cells pre-treated for 5–15 min with 100–200 μM 2′,5′-dideoxyadenosine, noradrenaline (10 μM) failed to affect steady-state pH_i ($n = 6/6$; not illustrated) and did not increase rates of pH_i recovery from imposed acid loads ($n = 7/7$; Fig. 9*F*). Finally, the ability of forskolin to increase steady-state pH_i was occluded by prior exposure to noradrenaline. Applied following a 15 min period of perfusion with 20 μM noradrenaline, which of itself increased pH_i by 0·23 ± 0·06 pH units ($n = 5$), 25 μM forskolin increased steady-state pH_i by only a further 0·02 ± 0·01 pH units. The results suggest that the effects of noradrenaline on pH_i are probably mediated via a signal transduction pathway which involves the activation of adenylate cyclase and the subsequent production of cAMP.

In the next series of experiments we examined the involvement of cAMP-dependent protein kinase (protein kinase A; PKA) in the pH_i response to β-adrenoceptor activation. Pre-treatment of neurones with the PKA inhibitors Rp-cAMPS (50 μM; Fig. 10*A*) and H-89 (10 μM; not illustrated) abolished the ability of 10 μM noradrenaline to increase steady-state pH_i ($n = 5/5$ and $n = 10/10$, respectively). In addition, both compounds inhibited the effect of 10 μM noradrenaline to increase rates of pH_i recovery following imposed acid loads ($n = 4/4$ and $n = 5/5$ for Rp-cAMPS (Fig. 10*B*) and H-89 (not illustrated), respectively). Conversely, a selective activator of PKA, Sp-cAMPS, mimicked the effects of noradrenaline by evoking a concentration-dependent rise in steady-state pH_i. Thus, applied at 5, 10, 25 and 40 μM, Sp-cAMPS caused steady-state pH_i to rise by 0·10 ± 0·01 ($n = 6/8$), 0·15 ± 0·01 ($n = 11/13$), 0·19 ± 0·01 ($n = 11/13$) and 0·20 ± 0·02 ($n = 2/2$) pH units, respectively (Fig. 10*C*); in the remaining neurones, Sp-cAMPS was without effect. The increase in steady-state pH_i evoked by 25 μM Sp-cAMPS under external Ca^{2+}-free conditions (a 0·20 ± 0·03 pH unit increase, $n = 3/3$) was not statistically different from that

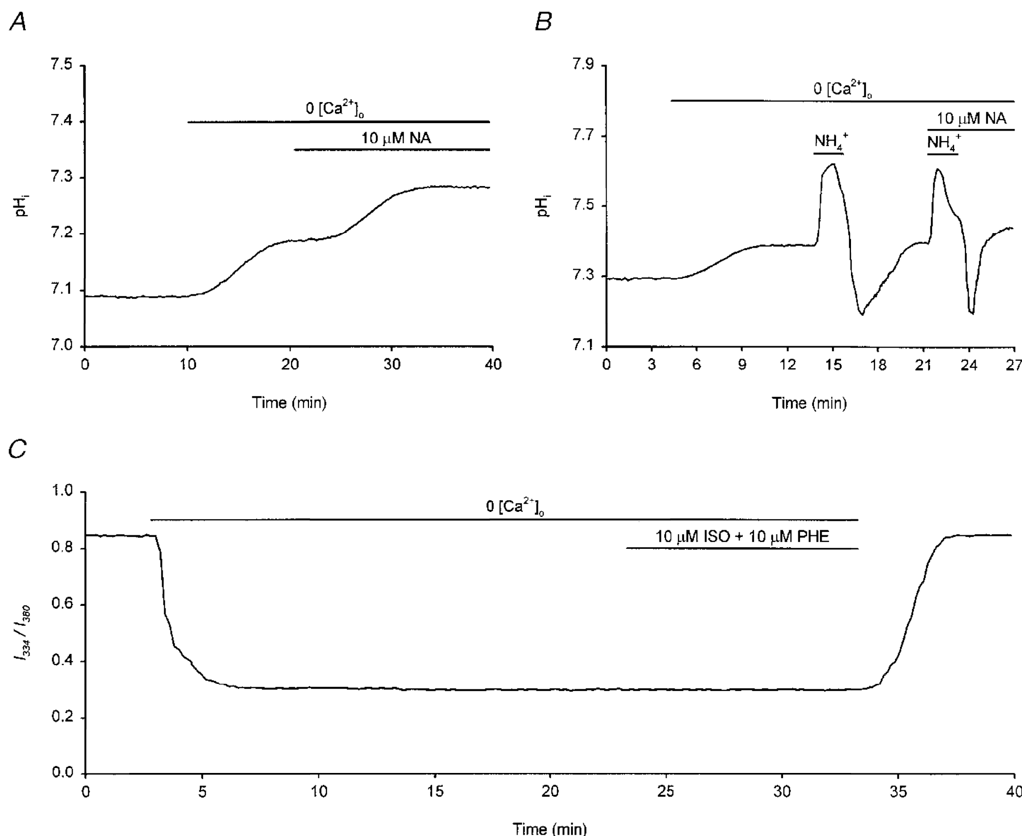

Figure 8. The pH_i response to noradrenaline is not dependent on changes in $[Ca^{2+}]_i$

A, exposure to a Ca^{2+}-free medium evoked an ~0·1 pH unit rise in resting pH_i. Once pH_i had stabilized at the new resting level, application of 10 μM noradrenaline (NA) elicited a further rise in pH_i. *B*, in a different neurone, removal of external Ca^{2+} again elicited a rise in steady-state pH_i. An initial intracellular acid load was then imposed and, following the recovery of pH_i, a second acid load was performed in the presence of 10 μM noradrenaline. Noradrenaline increased the rate of pH_i recovery in the absence of external Ca^{2+}. *C*, in a third neurone loaded with fura-2, exposure to Ca^{2+}-free medium caused a decrease in the I_{334}/I_{380} ratio value. Application of 10 μM isoprenaline (ISO, applied in the presence of 10 μM phentolamine (PHE)) did not evoke a change in the I_{334}/I_{380} ratio value. In contrast, like noradrenaline (see *B*), 10 μM isoprenaline increased steady-state pH_i under Ca^{2+}-free conditions (see text).

evoked in the presence of Ca^{2+}. Sp-cAMPS (25 μM) also increased the overall rate of pH_i recovery from imposed acid loads by 168 ± 24% in 9/10 neurones examined (Fig. 10*D*; in the remaining neurone, 25 μM Sp-cAMPS increased the overall rate of pH_i recovery by 33%) and shifted the pH_i dependence of acid extrusion in an alkaline direction by ~0·2 pH units (Fig. 10*E*). Finally, we examined whether inhibition of PKA could attenuate the ability of forskolin to increase steady-state pH_i. Applied following a 3–5 min period of pre-perfusion with 50 μM Rp-cAMPS, forskolin

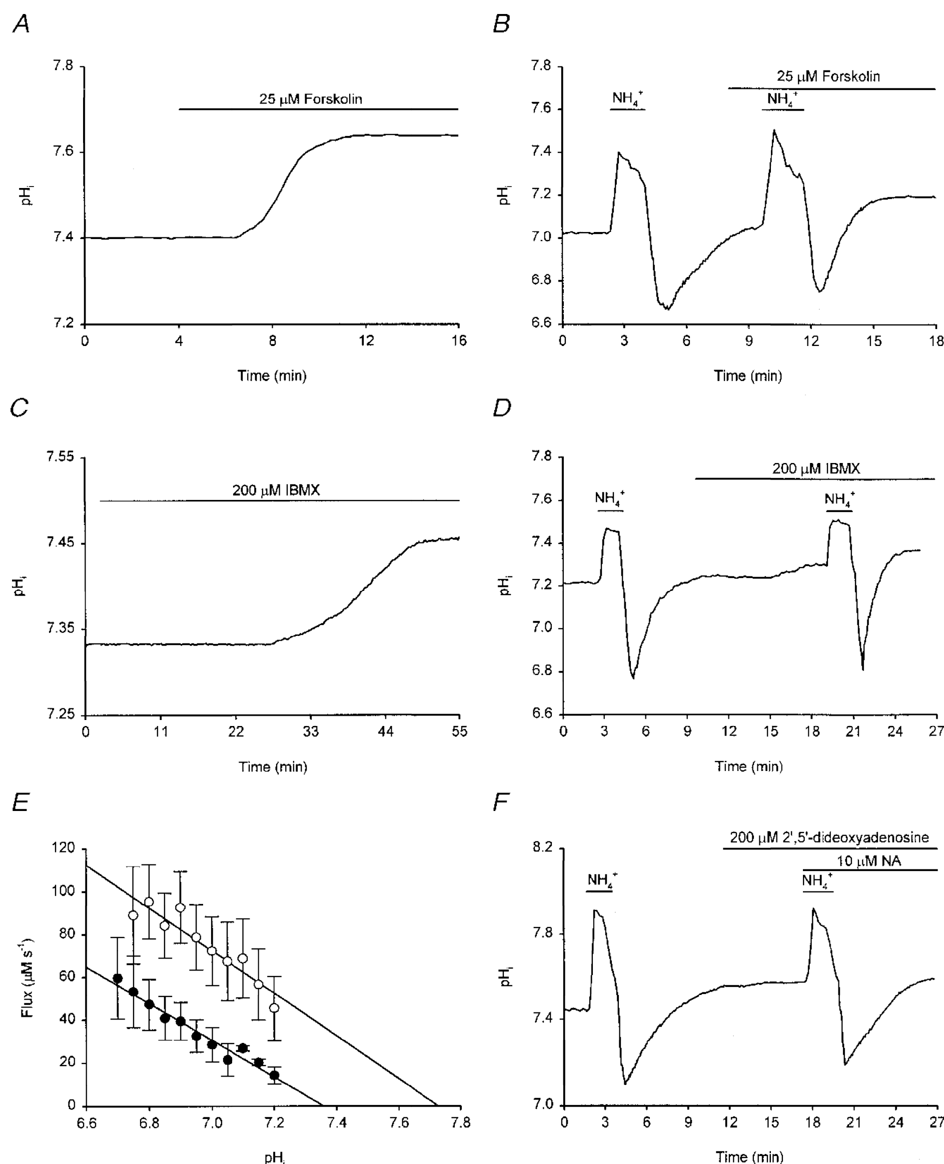

Figure 9. Involvement of cAMP in the pH_i response to noradrenaline

Records in *A*–*D* and *F* were obtained from different neurones. *A*, application of 25 μM forskolin evoked, after a short delay, a rise in steady-state pH_i. *B*, following an initial intracellular acid load, pH_i was allowed to recover. Forskolin (25 μM) was then applied and a second acid load was imposed. The rate of pH_i recovery was increased in the presence of forskolin and pH_i recovered to a new higher steady-state level. *C*, the phosphodiesterase inhibitor IBMX (200 μM) increased steady-state pH_i. *D*, an initial intracellular acid load was performed and, after pH_i had recovered, IBMX (200 μM) was applied. The rate of pH_i recovery following a second acid load was increased by IBMX and pH_i recovered to a new higher steady-state level. *E*, the pH_i dependence of net acid extrusion in the presence (○) and absence (●) of 25 μM forskolin. Continuous lines represent the least-squares linear regression fits to the data points indicated for each experimental condition. Data points were obtained from 5 experiments of the type illustrated in *B*; error bars represent S.E.M. Forskolin increased H^+ efflux ($P < 0.05$ at each absolute value of pH_i except pH_i 6·75, where $P = 0.23$) and shifted the pH_i dependence of acid extrusion to the right. *F*, following pre-treatment of the neurone with 200 μM 2′,5′-dideoxyadenosine, 10 μM noradrenaline failed to increase the rate of pH_i recovery from an imposed intracellular acid load.

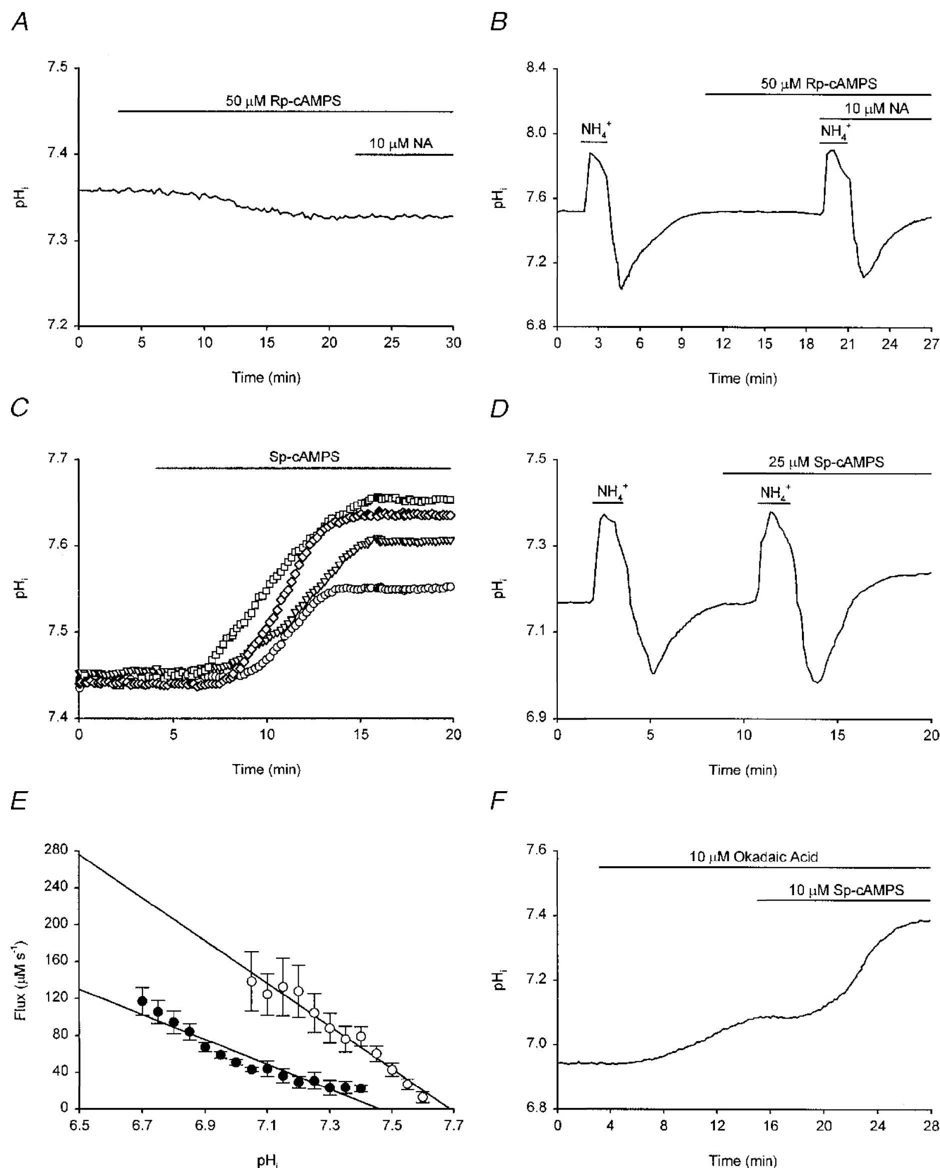

Figure 10. Involvement of cAMP-dependent protein kinase

Records in *A*–*F* were obtained from different neurones. *A*, following pre-treatment with 50 μM Rp-cAMPS for ~20 min, 10 μM noradrenaline (NA) failed to increase steady-state pH$_i$. *B*, an initial intracellular acid load was performed and, following the recovery of pH$_i$, 50 μM Rp-cAMPS was applied. A second acid load was then performed in the presence of 10 μM noradrenaline. Noradrenaline failed to increase the rate of pH$_i$ recovery from the imposed acid load following pre-treatment with Rp-cAMPS. *C*, Sp-cAMPS evoked a concentration-dependent rise in steady-state pH$_i$. Shown are the effects of 5 (○), 10 (▽), 25 (◇) and 40 μM Sp-cAMPS (□), applied to four different neurones with similar resting pH$_i$ values prior to the application of the compound. *D*, two intracellular acid loads were performed, the second in the presence of 25 μM Sp-cAMPS. The rate of pH$_i$ recovery was increased by Sp-cAMPS and pH$_i$ recovered to a new higher steady-state level. *E*, the pH$_i$ dependence of net acid extrusion in the presence (○) and absence (●) of 25 μM Sp-cAMPS. Continuous lines represent the least-squares linear regression fits to the data points indicated for each experimental condition. Data points were obtained from 9 experiments of the type illustrated in *D*; error bars represent S.E.M. Sp-cAMPS significantly ($P < 0.05$) increased H^+ efflux at each absolute value of pH$_i$ and shifted the pH$_i$ dependence of acid extrusion to the right. *F*, application of 10 μM okadaic acid evoked a rise in resting pH$_i$. Sp-cAMPS (10 μM) was then applied and caused resting pH$_i$ to increase to a greater extent than was observed in the absence of okadaic acid (see Results).

(25 μM) increased steady-state pH_i by 0·06 ± 0·04 pH units ($n = 10/10$; not shown), a change which was significantly smaller than that observed in the absence of Rp-cAMPS ($P < 0·005$).

The above results lead us to conclude that the effects of noradrenaline on steady-state pH_i and on acid extrusion following internal acid loads involve the activation of PKA by cAMP. The level of phosphorylation of acid extruding exchangers (and thus their activities) may result from an equilibrium between kinase and phosphatase activities (see Bianchini *et al.* 1991; Sardet *et al.* 1991). The possibility that phosphorylation of the Na^+–H^+ exchanger (or of an ancillary protein; see Discussion) might be involved in the pH_i response to PKA activation was suggested by experiments with the protein phosphatase inhibitor, okadaic acid. In HCO_3^--free medium containing 10 μM okadaic acid (Na^+ salt), which of itself produced a 0·18 ± 0·05 pH unit rise in pH_i, 10 μM Sp-cAMPS evoked a 0·36 ± 0·10 pH unit rise in pH_i in 4/4 neurones tested (Fig. 10*F*). This increase was significantly greater ($P < 0·01$) than the rise in pH_i evoked by 10 μM Sp-cAMPS in the absence of okadaic acid. We therefore suggest that a phosphorylation step may be involved in the effects of β-adrenoceptor activation on pH_i.

β-Adrenoceptors are coupled to adenylate cyclase activation through the α-subunit of the stimulatory G protein, G_s. If $G_{s\alpha}$ participates in the noradrenaline-evoked increase in

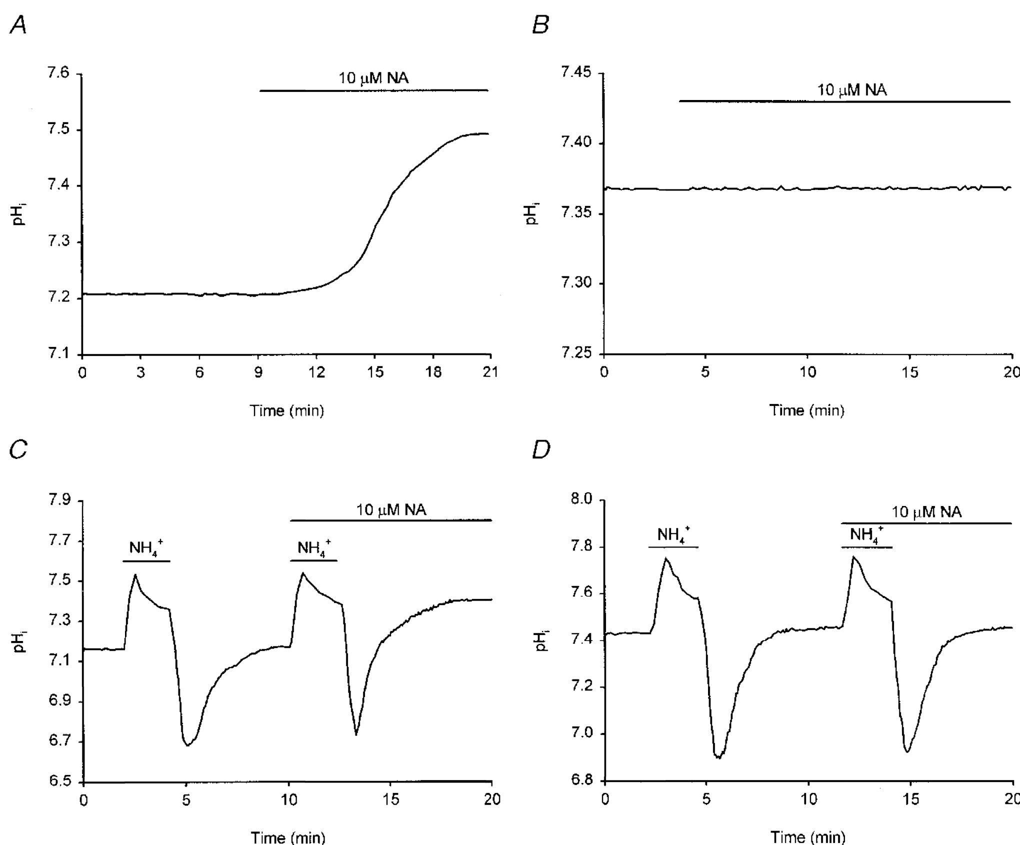

Figure 11. Pre-treatment with cholera toxin occludes the pH_i response to noradrenaline

A, noradrenaline (NA, 10 μM) was applied to a population of cultured postnatal rat hippocampal neurones and, after a short delay, pH_i increased to a new steady-state level (compare with Fig. 4*A*, the same experiment conducted in an acutely dissociated adult neurone). The trace represents the mean of data obtained from 18 neurones simultaneously. *B*, a sister culture to that employed in the experiment shown in *A* was pre-treated with 500 ng ml^{-1} cholera toxin for 18 h. Noradrenaline (10 μM) failed to increase steady-state pH_i. The trace represents the mean of data obtained from 21 neurones simultaneously. *C*, two consecutive intracellular acid loads were imposed on a third population of cultured postnatal hippocampal neurones, the second in the presence of 10 μM noradrenaline. The rate of pH_i recovery was increased by noradrenaline (compare with Fig. 4*C*, the same experiment conducted in an acutely dissociated adult neurone). The trace represents the mean of data obtained from 5 neurones simultaneously. *D*, a sister culture to that employed in the experiment shown in *C* was pre-treated with 500 ng ml^{-1} cholera toxin for 24 h. The overall rate of pH_i recovery from the second acid load conducted in the presence of 10 μM noradrenaline was comparable to the overall rate of pH_i recovery observed following the first acid load conducted in its absence. The trace represents the mean of data obtained from 10 neurones simultaneously.

Na^+–H^+ exchange activity, cholera toxin-catalysed ADP-ribosylation of $G_{s\alpha}$ should activate Na^+–H^+ exchange as if stimulated by noradrenaline and should not only increase steady-state pH_i but also occlude the effects of noradrenaline to increase steady-state pH_i and rates of pH_i recovery from acid loads. We therefore examined whether β-adrenoceptor-mediated regulation of acid extrusion proceeds through a pathway involving $G_{s\alpha}$ by pre-treating cultured postnatal rat hippocampal neurones with cholera toxin. Resting pH_i was $7{\cdot}33 \pm 0{\cdot}03$ in six neuronal cultures pre-treated with 500 ng ml^{-1} cholera toxin for 18–24 h, a value significantly higher than that measured in six sister cultures which had not been pre-treated with the toxin ($7{\cdot}18 \pm 0{\cdot}04$). In addition, in neurones pre-treated with cholera toxin, 10 μM noradrenaline failed to increase steady-state pH_i ($n = 3/3$; Fig. 11*B*) and did not affect the rate of pH_i recovery from imposed intracellular acid loads ($n = 3/3$; Fig. 11*D*). In contrast, in sister cultures which had not been pre-treated with cholera toxin, 10 μM noradrenaline increased resting pH_i by $0{\cdot}24 \pm 0{\cdot}03$ pH units ($n = 3/3$; Fig. 11*A*) and increased the overall rate of pH_i recovery from imposed acid loads by 126 ± 9% ($n = 3/3$; Fig. 11*C*). We therefore conclude that the effects of noradrenaline on pH_i are dependent upon the activation of $G_{s\alpha}$.

DISCUSSION

In acutely dissociated adult rat hippocampal CA1 neurones, noradrenaline evoked a concentration-dependent increase in steady-state pH_i by increasing the activity of a Na^+-dependent, HCO_3^--independent acid extrusion mechanism, probably the amiloride-insensitive variant of the Na^+–H^+ exchanger which has been extensively characterized in both fetal and adult rat hippocampal neurones (Raley-Susman *et al.* 1991; Schwiening & Boron, 1994; Baxter & Church, 1996; Bevensee *et al.* 1996). We found no evidence to suggest that the effect of noradrenaline to increase steady-state pH_i reflected changes in the activities of HCO_3^--dependent pH_i-regulating mechanisms. Nevertheless, it remains to be determined whether noradrenaline might modulate equally the activities of both HCO_3^--dependent acid loading mechanisms (e.g. Na^+-independent HCO_3^-–Cl^- exchange) and HCO_3^--dependent acid extruding mechanisms (e.g. Na^+-dependent HCO_3^-–Cl^- exchange), such that the resultant steady-state pH_i changes are no different from those found in the absence of HCO_3^- (see Ganz *et al.* 1989).

The effect of noradrenaline to increase both steady-state pH_i and net acid extrusion following imposed acid loads was mediated by β-adrenoceptors and proceeded via a pathway which probably involved a cholera toxin-sensitive G protein-coupled activation of adenylate cyclase and the subsequent stimulation of cAMP-dependent protein kinase. The evidence for this pathway is as follows. Firstly, the effects of noradrenaline on steady-state pH_i and acid efflux were mimicked by selective β_1- and β_2-adrenoceptor agonists, but not by a full α-adrenoceptor agonist, and were blocked by a full β-adrenoceptor antagonist. Secondly, incubation with cholera toxin occluded the effects of noradrenaline to increase both steady-state pH_i and rates of pH_i recovery following imposed acid loads. Thirdly, the effects of noradrenaline on both steady-state pH_i and acid extrusion were blocked by the adenylate cyclase inhibitor 2′,5′-dideoxyadenosine and were mimicked by forskolin and IBMX. Finally, pre-treatment with PKA inhibitors (Rp-cAMPS and H-89) blocked the effects of noradrenaline on steady-state pH_i and on rates of pH_i recovery from acid loads whereas an activator of PKA (Sp-cAMPS) mimicked the effects of noradrenaline on both parameters. In contrast to other cell types, we found no evidence to suggest either that β-adrenoceptor-mediated activation of Na^+–H^+ exchange in rat hippocampal neurones is independent of receptor coupling to G_s (Dhanasekaran *et al.* 1994; Voyno-Yasenetskaya *et al.* 1994) or that β-adrenoceptor stimulation regulates Na^+–H^+ exchange independently from a signalling cascade which involves cAMP (see Ganz *et al.* 1990). In addition, the regulatory pathway characterized in the present study differs from that described in other neuronal preparations. Thus, Na^+–H^+ exchange activity in rat brain synaptosomes is modulated by internal Ca^{2+} and not by protein kinases A or C (Sánchez-Armass *et al.* 1994). In contrast, in cerebellar Purkinje cells, Na^+–H^+ exchange is activated by protein kinase C (Gaillard & Dupont, 1990; the effect of modulating the cAMP/PKA pathway was not examined).

In peripheral cell types, it is well established that the activities of Na^+–H^+ exchangers are regulated by a wide variety of external stimuli that act via surface receptors linked to diverse intracellular signal transduction cascades. Furthermore, the response of Na^+–H^+ exchangers to a given stimulus appears highly dependent not only on the particular isoform of the exchanger being studied but also on the cell type or cell line in which the particular isoform is expressed (see Wakabayashi *et al.* 1997). Indeed, the adrenoceptor subtype and intracellular pathway which couples activation of the receptor to the Na^+–H^+ exchanger in rat hippocampal neurones appear to differ from those involved in many other preparations. Thus, in the majority of cell types studied to date, α-adrenoceptors mediate the stimulatory effects of adrenergic receptor agonists on Na^+–H^+ exchange. In cardiac myocytes, for example, α_1-adrenoceptor stimulation increases steady-state pH_i through protein kinase C-mediated activation of Na^+–H^+ exchange whereas β-adrenoceptor agonists either fail to influence exchange activity or inhibit it (e.g. Guo *et al.* 1992; Wallert & Fröhlich, 1992; Lagadic-Gossman & Vaughan-Jones, 1993). Furthermore, elevating $[cAMP]_i$ or activating PKA either has no effect on or inhibits Na^+–H^+ exchange (NHE) activity in most cell types studied (e.g. Wu & Vaughan-Jones, 1994; Kurashima *et al.* 1997), although there are some exceptions (reviewed by Wakabayashi *et al.* 1997; see also Kandasamy *et al.* 1995). Perhaps the best-characterized example of a cAMP-mediated upregulation of Na^+–H^+ exchange activity is seen in the case of the β-NHE

isoform, which can be activated both by β-adrenoceptor agonists and by direct elevation of $[cAMP]_i$ (see Noël & Pouysségur, 1995). Interestingly, β-NHE contains two potential consensus sites for phosphorylation by PKA in its C-terminal cytoplasmic domain (Borgese *et al.* 1992). Although it is unclear precisely which NHE isoform participates in pH_i regulation in rat hippocampal CA1 neurones, it is tempting to speculate that it might also possess an intrinsic capability to respond to PKA.

Activation of Na^+–H^+ exchange by mitogens, hormones and other external agents occurs through an increased affinity of the allosteric internal modifier site on the exchanger for protons and/or an increase in the maximum velocity of transport (see Mahnensmith & Aronson, 1985; Noël & Pouysségur, 1995). The former process, which may reflect a conformational change in the transport protein produced by phosphorylation, results in an alkaline shift in the pH_i dependence of the exchanger. The possibility that a similar mechanism might underlie the effects of β-adrenoceptor activation observed in the present study is suggested by the rightward shifts in the pH_i dependence of acid extrusion evoked by noradrenaline (Fig. 4*D*), β-adrenoceptor agonists (Fig. 5*D*), forskolin (Fig. 9*E*) and Sp-cAMPS (Fig. 10*E*). Furthermore, the ability of the protein phosphatase inhibitor okadaic acid to augment the increase in pH_i evoked by a PKA activator (Fig. 10*F*) suggests that, as in other cell types (see Bianchini *et al.* 1991; Sardet *et al.* 1991; Wakabayashi *et al.* 1997), a phosphorylation event might be involved in modifying the mechanistic properties of the exchange mechanism in hippocampal neurones. However, we have no evidence to indicate whether activation of Na^+–H^+ exchange by PKA involves direct phosphorylation of the exchange protein itself or of an associated regulatory protein whose phosphorylation state influences the former's activity (see Sardet *et al.* 1991; Lin & Barber, 1996; Yun *et al.* 1997; Hall *et al.* 1998).

The functional significance of the present findings remains unclear. Nevertheless, the magnitude of the effect of noradrenaline on steady-state pH_i is consistent with the possibility that, as in peripheral cell types, adrenoceptor-mediated modulation of the activity of neuronal acid extrusion mechanisms may represent a physiologically relevant transmembrane signalling pathway. In this regard, a number of speculative possibilities exist. Firstly, as noted in the Introduction, neuronal ionic conductances (Daumas & Andersen, 1993; Tombaugh & Somjen, 1997) and the activities of intracellular enzymes (e.g. Vignes *et al.* 1996) are sensitive to shifts in pH_i, as are the activities of transport and buffering mechanisms for various ions (e.g. Zucker, 1981; Dipolo & Beaugé, 1982; Sidky & Baimbridge, 1997). Although the net functional result of changes in pH_i on all of these processes is impossible to predict, β-adrenoceptor-mediated changes in steady-state pH_i might contribute to at least some of the complex effects of β-adrenoceptor stimulation or activation of the cAMP/PKA signalling cascade on hippocampal neuronal function. The possible participation of increases in pH_i in some of the more persistent effects of β-adrenoceptor activation or $[cAMP]_i$ elevation, such as increases in excitatory synaptic strength (Dunwiddie *et al.* 1992; Raman *et al.* 1996) and facilitation of the induction of long-term potentiation (Thomas *et al.* 1996; Bolshakov *et al.* 1997), is especially attractive given that changes in pH_i are known to affect processes such as gene expression, protein synthesis and cytoskeletal reorganization in peripheral cell types (reviewed by Busa & Nuccitelli, 1984; Grinstein *et al.* 1989). Secondly, the increase in acid efflux evoked by β-adrenoceptor activation may provide a mechanism to rapidly alleviate intracellular acid loads occasioned by neuronal activity and the application of neurotransmitters such as glutamate (see Introduction). Thirdly, changes in pH_i consequent upon β-adrenoceptor activation will inevitably affect the pH of the microenvironment. The changes in pH_o may, in turn, affect not only the activities of pH_i-regulating mechanisms in adjacent cells but also neuronal excitability, given the established sensitivity of neuronal voltage- and ligand-activated ion channels to changes in pH_o (e.g. Vyklický *et al.* 1990; Tombaugh & Somjen, 1996). Finally, in light of the fact that activation of Na^+–H^+ exchange contributes to neuronal death following metabolic inhibition (see Vornov *et al.* 1996), it is possible that β-adrenoceptor-mediated stimulation of acid extrusion participates in β-adrenoceptor-mediated potentiation of ischaemia-induced neuronal damage (e.g. Shibata *et al.* 1992).

In summary, noradrenaline increases steady-state pH_i in acutely dissociated adult rat hippocampal CA1 neurones. The effect, which is mediated by β-adrenoceptors, is probably due to activation of the acid-extruding Na^+–H^+ exchanger via a pathway involving G_s, cAMP and PKA. The exact relationship between PKA activation and stimulation of the transport mechanism, however, remains to be determined. In many peripheral cell types, the activities of Na^+–H^+ exchangers can be controlled independently by multiple external stimuli acting via different signalling pathways (see Borgese *et al.* 1992; Kandasamy *et al.* 1995; Noël & Pouysségur, 1995; Wakabayashi *et al.* 1997). Given that pH_i is an important determinant of neuronal function, it will be of interest to examine whether additional external stimuli and intracellular signal transduction cascades might be involved in the regulation of the activities of the acid extrusion mechanisms present in rat hippocampal neurones.

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