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Modulation of the Glutamate-Evoked Release of Arachidonic Acid from Mouse Cortical Neurons: Involvement of a pH-Sensitive Membrane Phospholipase A_2

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Excitatory synaptic transmission is associated with changes in both extracellular and intracellular pH. Using mouse cortical neurons in primary cultures, we studied the sensitivity of glutamate-evoked release of ^{3}H-arachidonic acid (^{3}H-AA) to changes in extracellular pH (pH_o) and related intracellular pH (pH_i). As pH_o was shifted from 7.2 to 7.8, the glutamate-evoked release of ^{3}H-AA was enhanced by ~ threefold. The effect of alkaline pH_o on the glutamate response was rapid, becoming significant within 2 min. ^{3}H-AA release, evoked by both NMDA and kainate, was also enhanced by pH_o alkalinization. NMDA- and kainate-induced increase in free intracellular Ca^{2+} was unaffected by changing pH_o from 7.2 to 7.8, indicating that the receptor-induced Ca^{2+} influx is not responsible for the pH_o sensitivity of the glutamate-evoked release of ^{3}H-AA.

Alkalinization of pH_i obtained by incubating neurons in the presence of HCO_3^- or NH_4 enhanced the glutamate-evoked release of ^{3}H-AA, while pH_i acidification obtained by blockade of Na^+/H^+ and Cl^-/HCO_3^- exchangers decreased the glutamate response. Membrane-bound phospholipase A_2 ($mPLA_2$) activity was stimulated by Ca^{2+} in a pH-dependent manner, increasing its activity as pH was shifted from 7.2 to 7.8. This pH profile corresponds to the pH profile of the glutamate-, NMDA- and kainate-evoked release of ^{3}H-AA. Taken together, these results indicate that the glutamate-evoked release of ^{3}H-AA may be mediated by the pH-sensitive $mPLA_2$. Since excitatory neurotransmission mediated by glutamate results in both pH_o and pH_i changes and since AA enhances glutamatergic neurotransmission at both pre- and postsynaptic levels, the data reported here reveals a possible molecular mechanism whereby glutamate can modulate its own signalling efficacy in a pH-dependent manner by regulating the release of AA.

[Key words: H^+, NMDA, kainate, intracellular Ca^{2+}, long-term potentiation, ischemia]

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Excitatory synaptic transmission is associated with both extracellular and intracellular changes in pH homeostasis. Extracellular pH (pH_o) shifts to alkaline values (up to 0.4 pH units) for several minutes after a brief electrically evoked depolarization (Kraig et al., 1983; Chesler and Chan, 1988). It has been proposed that pH_o changes are due to the exchange of H^+ equivalents principally between the interstitial space and surrounding glial cells (Chesler, 1990; Ransom, 1992). In line with this hypothesis, Bouvier et al. demonstrated that glutamate uptake into glial cells is associated with the countertransport of OH^- to the extracellular medium (Bouvier et al., 1992). Thus, extracellular alkalinization could result from this neuronal–glial interaction due to glutamate reuptake into astrocytes. Glutamatergic neurotransmission can also induce intracellular pH (pH_i) changes in neuronal cells, since rapid pH_o shifts induce pH_i shifts in the same direction within seconds (Ou-yang et al., 1993). In addition, activation of NMDA receptors modifies intracellular H^+ homeostasis to alkaline values that last up to several hours (Hartley and Dubinsky, 1993). Thus, glutamatergic transmission is associated with complex and long-lasting changes in both pH_o and pH_i. Such shifts in pH homeostasis could, in turn, modify the glutamate-mediated neurotransmission by affecting either glutamate transport (Tabb et al., 1992), glutamate receptors, or the signal transduction pathways associated with the activation of these receptors. For example, pH_o-sensitive NMDA currents have been characterized (Tang et al., 1990; Traynelis and Cull-Candy, 1990).

Activation of glutamatergic receptors induces the formation of second messengers. In particular, glutamate evokes the release of arachidonic acid (AA) from hippocampal slices (Pellerin and Wolfe, 1991), as well as from both neurons and astrocytes in primary culture (Dumuis et al., 1988, 1990; Lazarewicz et al., 1988; Sanfeliu et al., 1990; Tapia-Arancibia et al., 1990, 1992; Stella et al., 1994). This fatty acid is released from the *sn*-2 position of membrane phospholipids by the activation of various phospholipases, including phospholipases A_2 (PLA_2) (Irvine, 1982; Dennis, 1994). Interestingly, brain synaptic vesicular PLA_2 activity has been shown to be pH sensitive (Moskowitz et al., 1983). Taken together, this array of experimental evidence raises the question of whether changes in either pH_o or pH_i would affect the glutamate-evoked release of AA. A pH sensitivity of the glutamate-evoked release of AA could have some relevance to the physiological regulation of glutamate neurotransmission, since it has been previously reported that AA itself modulates glutamate neurotransmission. Indeed, AA enhances

NMDA currents (Miller et al., 1992), possibly by interacting with the ion channel protein itself (Petrou et al., 1993) and it may enhance the concentration of glutamate in the synaptic cleft by both increasing glutamate release from presynaptic terminals (Herrero et al., 1992) and by inhibiting glutamate reuptake (Yu et al., 1986; Barbour et al., 1989; Volterra et al., 1992).

Thus, the fine regulation of glutamate-evoked release of AA by either pH_o or pH_i would add yet another level of complexity to the feedback control of AA on glutamatergic neurotransmission. In this article, we used primary cultures of cerebral cortical neurons totally devoid of glial cells to characterize the glutamate-evoked release of AA. We show that glutamate stimulates AA release in a pH-dependent manner and describe the cascade of events that leads to this action. The data presented reveal a molecular mechanism whereby glutamate neurotransmission could regulate its own signalling efficacy.

Materials and Methods

Materials

Poly-L-ornithine (MW, 30,000–70,000), Dulbecco's modified Eagle's medium (DMEM; D-7777), bovine pancreas insulin, human apo-transferrin, putrescine, progesterone, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyletrazolium bromide (MTT), fatty acid free bovine serum albumin (BSA), L-glutamate, L-aspartate, L-homocysteate, NMDA, kainate, D-serine, concanavalin A, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), harmaline, and amiloride were obtained from Sigma, St.-Quentin-Fallavier, France; (*RS*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (*t*-ACPD), L(+)-2-amino-3-phosphonopropionic acid (AP3), L-cysteate, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 6,7-dinitroquinoxaline-2,3-dione (DNQX) from Tocris Neuramin, Bristol, England; 5*R*,10*S*-(+)-5-methyl-10,11-dihydro-5-H-dibenzo [a,d] cyclohepten-5,10-imine hydrogen maleate (MK801) and D-2-amino-5-phosphonopentanoic acid (AP5) from Research Biochemicals Incorporated, Natick, MA; adenosine deaminase (EC 3.5.44) from Boehringer, Mannheim, Germany; PTX from List, Campell, CA; myo-[2-^{3}H]-inositol with PTG-271 [633 GBq (17.1 Ci)/mmol], ^{3}H-arachidonic acid (^{3}H-AA, 8.25 TBq (200 Ci)/mmol), and ^{14}C-arachidonic acid [^{14}C-AA, 2.15 GBq (58 mCi)/mmol] from Amersham, Buckinghamshire, England; INDO-1 AM, 1,2-bis-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (PPC); pyrenedecanoate from Molecular Probes, Eugene, OR.

Methods

Cell culture. To obtain cultures devoid of glial cells, cortical neurons were prepared as described by DiPorzio and colleagues by taking advantage of the fact that non-neuronal cells do not proliferate in the absence of serum (DiPorzio et al., 1980). Accordingly, Swiss Albino timed-pregnant mice (Iffa Credo, Lyon, France) were killed by decapitation and 16-d-old embryos were removed. The embryos were decapitated and their heads were washed six times in a phosphate-buffered saline supplemented with 33 mM glucose (PBSglc). The cerebral cortex was dissected by removing the olfactory lobe, striatum, hippocampus, and meninges. Cells were dissociated mechanically through a flame-narrowed Pasteur pipette by two series of 15–20 gentle flushes in PBSglc (first in 5 ml and then in 12 ml). Debris were removed by three centrifugations at 200 × *g* for 5 min. Cells (800,000 cells/ml) were plated on either 12-well Falcon culture dishes (1 ml/well) or 90 mm dishes (15 ml/dish), previously coated for at least 2 hr with 15 μg/ml polyornithine, rinsed once with water and then once with PBSglc. For intracellular Ca^{2+} ($[Ca^{2+}]_i$) imaging, cells were plated on coverslips also coated with polyornithine, followed by 2 μg/ml laminin for at least 2 hr. In all cases, coating was carried out in serum-free medium. The medium was composed of DMEM supplemented with 2 mM glutamine, 7.5 mM $NaHCO_3$, 5 mM HEPES buffer (pH 7.0), 100 μg/ml streptomycin, and 60 μg/ml penicillin. A mixture of hormones and salt containing 25 μg/ml insulin, 100 μg/ml transferrin, 60 μM putrescine, 20 nM progesterone, and 30 nM sodium selenate was added to the culture medium. Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Characterization of cell viability as a function of days in vitro. Neurons cultured in the total absence of serum have a limited viability with increasing days *in vitro* (DIV) (DiPorzio et al., 1980). Thus, we characterized the viability of neurons in culture as a function of increasing DIV by measuring the cleavage of MTT into formazan as described by Denizot and Lang (1986). This reaction occurs only in living cells and is directly proportional to the activity of mitochondrial dehydrogenases (Slater et al., 1963). Briefly, cortical neurons cultured in 12-well culture dishes for increasing DIV were incubated for 3 hr with MTT at a final concentration of 0.5 mg/ml added directly to the culture medium (100 μl/well of a solution containing 5 mg MTT/ml PBS). At the end of the incubation, the medium was removed and the precipitate was dissolved with 1 ml dimethyl sulfoxide. Dishes were then shaken and optical density was measured at 560 nm. Optical density of dissolved formazan reached a maximum between 6 and 7 DIV (Fig. 1). Therefore, in following experiments, cortical neurons were studied at 6 ± 1 DIV.

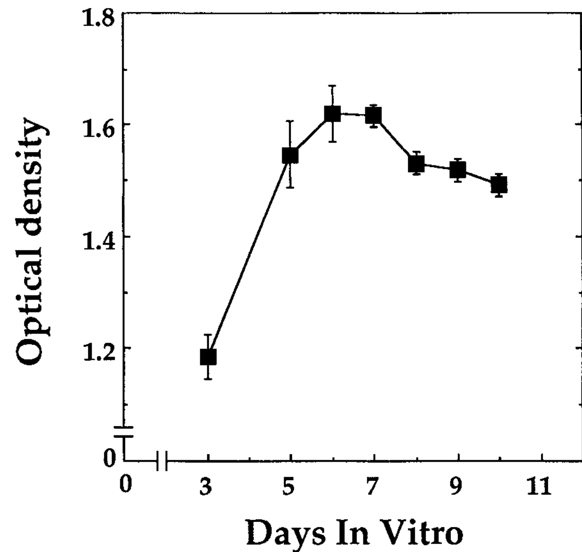

Figure 1. Viability of cortical neurons as a function of DIV. Cortical neurons at increasing DIV were incubated for 3 hr in the presence of MTT, and formazan was measured as described in Materials and Methods. Results are the mean ± SEM of three separate determinations from one experiment repeated twice with similar results.

Immunocytochemical characterization. Cells grown for 6 DIV on glass coverslips (Deckgläser, Germany) in 12-well dishes were rinsed once with Dulbecco's phosphate-buffered salin (Dulbecco's PBS; pH 7.5; GIBCO, Eragny, France) at room temperature and fixed with 4% paraformaldehyde for 15 min. Each well was rinsed four times with Dulbecco's PBS and 10% normal goat serum (Nordic Immunology) was then added for 30 min to the cells before applying the different monoclonal antibodies for 18 hr at 4°C. Antisera directed against neuronal-specific enolase (1:200; kindly provided by N. Lamandé, Collège de France, Paris, France; Secchi et al., 1980) and against neurofilament (1:1000; SMI31; Sternberger, Baltimore, MD; Dahl, 1988) were used as neuronal markers. Antisera directed against glial fibrillary acidic protein (1:200, GFAP; ICN, Costa Mesa, CA), galactocerebroside (1/100; Boehringer), and MAC1 (1:10; Serotec, Oxford, England; Frei et al., 1987) were used as markers for astrocytes, oligodendrocytes, and microglia, respectively. For surface marker staining, cells were rinsed three times with Dulbecco's PBS containing 10% normal goat serum, whereas 0.1% Triton was also added for intracellular markers. Characterization was performed using indirect immunofluorescence staining by adding the secondary antibody for 1 hr at room temperature. Cells were observed under a Nikon Optiphot microscope. Cells in the culture exclusively expressed neuronal markers, that is, neuronal-specific enolase and neurofilament (94.3 ± 1.2% and 98.8 ± 0.8%, respectively; both total and stained cells were counted: $n = 10$; see Fig. 2*A*,*B*). No detectable staining was found for astrocytes, oligodendrocytes, or microglia, using GFAP, galactocerebroside, and MAC1 antibodies, respectively (Fig. 2*C*,*D*,*E*). Cortical neurons cultured up to 11 DIV were still GFAP negative (not shown).

Neuronal viability during acute glutamate application. It is well established that glutamate can be neurotoxic, depending on DIV or on glial density (Choi et al., 1987; Rosenberg et al., 1992). Therefore, we

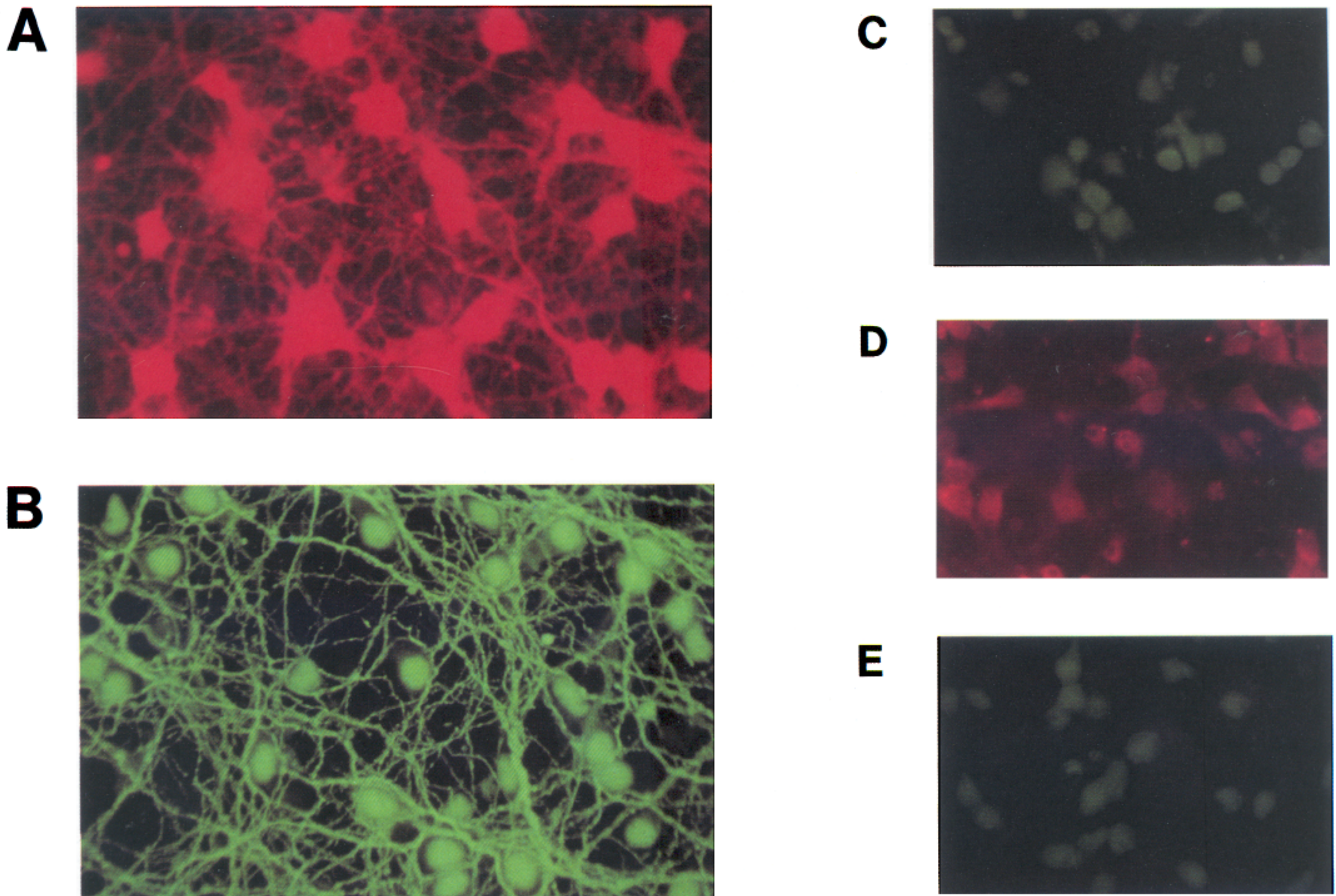

Figure 2. Immunocytostaining of cortical neurons. Six DIV neurons originating from 16-d-old embryonic mouse cerebral cortex were cultured in the total absence of serum and immunocytostained as described in Materials and Methods. Immunocytostaining for neuron specific enolase (*a*), neurofilaments (*b*), GFAP (*c*), Gal C (*d*), and Mac1 (*e*) are shown.

investigated the potential neurotoxicity of glutamate by using Trypan blue as an index of plasma membrane permeation due to cell death. Application of 100 μM glutamate to cortical neurons in primary culture for 15 min (i.e., the same conditions as those used for ^{3}H-arachidonic acid (^{3}H-AA) release; see below), followed by addition of Trypan blue (0.01% v/v) revealed no difference in Trypan blue exclusion after this treatment when compared with untreated control neurons (not shown).

Measurement of ^{3}H-arachidonic acid release. ^{3}H-AA release was measured according to the method described by Lazarewicz et al. (1988), with minor modifications. Briefly, cortical neurons cultured in 12-well culture dishes were incubated for 18 hr in the presence of ^{3}H-AA (1 μCi/50 μl PBSglc + 0.1% BSA/well) added to the culture medium. Under these conditions, cells incorporated 85–90% of the added radioactivity. When stated, cells were treated with PTX added to the culture medium during the labeling period. After washing three times at 37°C with a Locke-HEPES buffer (L-H buffer; in mM: NaCl, 145; KCl, 5.5; $CaCl_2$, 1.1; $MgCl_2$, 1.1; $NaHCO_3$, 3.6; glucose, 5.5; HEPES, 20; pH, 7.4; 1 ml/well) containing 2 mg/ml BSA, cells were preincubated for 15 min in the same medium in the presence of adenosine deaminase (1 IU/ml). This latter procedure was used to degrade endogenous adenosine that has been previously shown to affect glutamate-mediated signal transduction (El-Etr et al., 1992). Unless otherwise stated, inhibitors or antagonists were also added during this preincubation period. Cultures were then exposed for 15 min at 37°C to the agents added at final concentration in the same medium (pH 7.2–7.8, as specified), but lacking Mg^{2+} (a condition necessary to yield full activity of NMDA receptors; Nowak et al., 1984; Mayer et al., 1984). At the end of the incubation, the medium was collected and centrifuged at 200 × *g* for 5 min to remove contamination by occasionally present detached cells. An aliquot of the supernatant was assayed for ^{3}H by liquid scintillation counting. Agonist-induced release into the extracellular medium corresponds to 1–3% of cellular ^{3}H content (see also Dumuis et al., 1988). The protein content of the cultures was determined by the method of Bradford (1976).

Thin-layer chromatography analysis of ^{3}H-arachidonic acid release. In similar cortical neurons in primary cultures, it has been shown by reverse-phase HPLC that the radioactivity released into the incubation medium after glutamate stimulation is recovered in a single peak with a retention time identical to AA (Oomagari et al., 1991). Confirming these results, thin-layer chromatography (TLC) characterization of the ^{3}H labeled product(s) released from cortical neurons further support the conclusion that glutamate enhanced exclusively ^{3}H-AA release. Briefly, a fixed amount of ^{14}C-AA (internal standard) was added to 1.5 ml of supernatant containing the released ^{3}H-labeled product(s). Lipids were extracted by standard Folch extraction. Thus, $CHCl_3/CH_3OH$ (2:1 v/v) was added to the supernatant to obtain a final proportion of $CHCl_3/CH_3OH/H_2O$ of 8:4:3. After complete phase separation, the lower phase was recovered, evaporated to dryness, and the final residue dissolved in 50 μl of $CHCl_3/CH_3OH$ (2:1 v/v). Samples, as well as nonradioactive standards were plated on dried TLC silica gel plates (20 × 20 cm; 250 μm thickness; Sigma), and separation was obtained by developing the plates in benzene/dioxan/acetic acid (60:40:2 by volume) as the mobile phase. This solvent system was shown to allow complete separation of AA from other classes of lipids such as phospholipids, mono- and diacylglycerols, as well as its own metabolites including prostaglandins and most lipoxygenase metabolites (Hurst et al., 1987). Nonradioactive standards, including AA, were visualized by spraying the TLC plate with phosphomolybdic acid (Sigma) and heating it by blowing hot air. Areas corresponding to each lipid class for each sample were scraped and ^{3}H and ^{14}C were assayed by liquid scintillation counting. Each value was corrected according to the recovery of internal standard, which was greater than 92% in all cases.

Measurement of ³H-inositol phosphate formation. Cells were grown in the same conditions for 6 DIV in the presence of myo-[2-³H]-inositol (4 μCi/ml). The accumulation of ³H-inositol phosphates (³H-IPs) was determined as described by (Berridge et al., 1982) with minor modifications. Briefly, cultures were washed three times with L-H buffer (1 ml/well) at 37°C, and subsequently preincubated for 15 min in L-H buffer supplemented with lithium (10 mM) and adenosine deaminase (1 IU/ml). Cultures were then exposed for 15 min at 37°C to the agents added at a final concentration in a Mg^{2+}-free L-H buffer. The incubation was stopped by lysing the cells with successive addition of 0.1% Triton X-100 in 0.1 M NaOH (400 μl) and 0.1% Triton X-100 in 0.1 M HCl (400 μl). ³H-IPs contained in the lysate was isolated by addition of 1.5 ml $CHCl_3/CH_3OH$ (2:1 v/v), followed by 0.5 ml $CHCl_3$ and by a centrifugation at 1000 × *g* for 5 min. An aliquot (1 ml) of the upper aqueous phase was loaded onto Dowex AG 1-×8 columns (formate form, 200–400 mesh, Bio-Rad) and myo-[2-³H]-inositol was eluted with myo-inositol (5 mM, 4 ml). Columns were then washed with formic acid (0.1 M, 10 ml) and total ³H-IPs, containing mainly ³H-monophosphate (more than 90% of total IPs; El-Etr et al., 1989) were eluted with 5 ml ammonium formate (1 M)/formic acid (0.1 M). Radioactivity was measured by adding H_2O (3 ml) and Aquasol 2 (8 ml).

Intracellular $[Ca^{2+}]$ determination. Determination of $[Ca^{2+}]_i$ was carried out as previously described, with minor modifications (Marin et al., 1993). Briefly, cortical neurons grown on coverslips were studied with a dual emission microfluorimetry using the fluorescent dye INDO-1 AM. Cells were loaded for 60 min in the presence of 12 μM of INDO-1 AM in Locke-HEPES buffer. After loading, cells were exposed to various substances dissolved in Locke-HEPES buffer using a multichannel cell superfusion device allowing the complete change of the superfusion medium in less than 0.2 sec. All responses were measured in the absence of Mg^{2+}. In addition, NMDA responses were examined in the presence of D-serine (100 μM) to yield full NMDA receptor activation on perfused neurons (Johnson and Ascher, 1987). Cells were excited with a 75 W Xenon light filtered at 340 nm with a 10 nm wide interference filter. Excitation and emission spectra were separated by a 380 nm dichroic long-pass filter and the emission spectra were then divided in two halves by a dichroic long pass filter (Opticals were from Nikon). Two discriminant bands were selected from the two halves at 400–410 nm and 470–480 nm, and both fluorescent images were digitized (8 video frames/digitized image). The camera dark noise was subtracted from the recorded crude image (camera and digitized system were from Hamamatsu Ltd., Japan).

Calculation of intracellular $[Ca^{2+}]$ changes. The concentration of $[Ca^{2+}]_i$ (nM) was calculated from the fluorescence ratio (R) measured at 400–410 nm and 470–480 nm, according to the equation described by Grynkiewicz et al. (1985): $[Ca^{2+}]_i = K_d \times (F_{480f}/F_{480b}) \times (R - R_{min})/(R_{max} - R)$, where the K_d of INDO-1 for ionized Ca^{2+} is 250 nM, F_{480f} is the fluorescence of free INDO-1, F_{480b} the fluorescence of INDO-1 bound to Ca^{2+}, and R is the ratio between fluorescences measured at 405 and 480 nm. R_{max} and R_{min} was determined in the presence of ionomycin (5 μM), with 1.1 mM $CaCl_2$ or 2 mM EGTA, respectively. $[Ca^{2+}]_i$ was measured at 2.5 sec intervals in the cell bodies of individual neurons. $[Ca^{2+}]_i$ plateau values were expressed as the mean of the three highest and three lowest values observed during the application of agents to *n* cells. Only data obtained during the first stimulation with the agonist were considered for calculations. Ninety-three and 92% of cells tested responded to NMDA + D-serine or kainate, respectively.

PLA_2 assay. PLA_2 activity was determined according to the method described by Piomelli and Greengard (1991), with minor modifications. Briefly, cortical neurons prepared in 90 mm dishes were incubated under the same conditions as described for the ³H-AA release. The incubation was stopped by removing the buffer and by adding 1 ml ice-cold hypotonic buffer (lysis buffer) containing 1 mM EDTA, 1 mM EGTA, 10 mM sodium pyrophosphate, 10 mM HEPES, 5 μg/ml trypsin inhibitor (Sigma), and 0.1 μM phenylmethylsulfonyl fluoride. Lysed cells were scraped and dishes were rinsed with 1 ml of the same lysis buffer to recover remaining cellular elements. The lysate was centrifuged for 15 min at 40,000 × *g* to separate cytoplasm from plasma membrane. The fluorogenic PLA_2 substrate PPC (2 μM) was added to 1.5 ml of 0.1 M Tris-HCl (at indicated pH) in a stirred quartz cuvette maintained at 37°C within a F2000 Hitachi fluorimeter (excitation wavelength, 340 nm; emission wavelength, 380 nm). It should be noted that the PPC spectrum was insensitive to pH over the range used in the present study (not shown). After 3–4 min (fluorescence stabilization), a sample of either cytoplasm or membrane (25 μg of protein) was transferred into the cuvette and the rate of increase in fluorescence due to the release of pyrenedecanoic acid was monitored every 5 sec over a 2 min period. This measure represents the stable activity of the Ca^{2+}-independent PLA_2. Ca^{2+}-dependent PLA_2 activity was determined by adding Ca^{2+} (2 mM final concentration) on a period of 2–5 min, depending on the experiments. The changes in fluorescence due to released pyrenedecanoate were calibrated against a known concentration of the unesterified fatty acid.

Statistical analysis. Results are expressed as mean ± SEM of *n* independent determinations. Data were statistically analyzed using INSTAT, GraphPad Software, San Diego, CA.

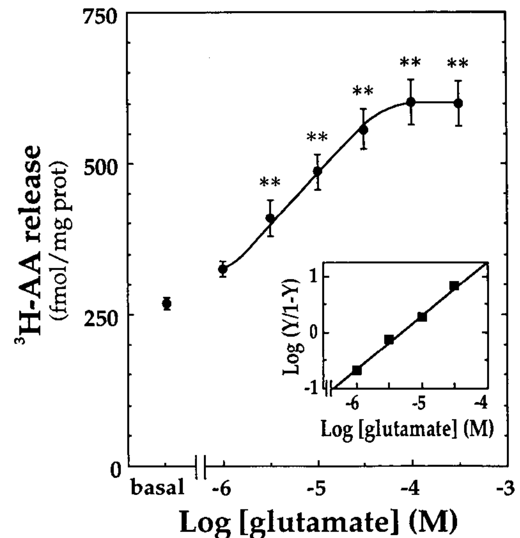

Figure 3. Glutamate evokes a concentration-dependent release of ³H-arachidonic acid. Cortical neurons were incubated for 15 min with increasing concentrations of glutamate. ³H-AA release was determined as described in Materials and Methods. Results, expressed as fmol per mg of protein, are mean ± SEM of 18 separate determinations for each concentration resulting from six different experiments. **, $p < 0.01$ compared to basal ³H-AA release (ANOVA, followed by Dunnett's test). *Inset*, Hill plot of the concentration-dependent glutamate-evoked release of ³H-AA.

Results

Characterization of the excitatory amino acid induced ³H-arachidonic acid release

As previously demonstrated, both basal and 100 μM glutamate-evoked release of ³H-AA increased rapidly during the first 10 min of incubation to then reach a plateau (not shown, see Dumuis et al., 1988). Therefore, a 15 min incubation was used to investigate the effects of pharmacological agents on ³H-AA release. Glutamate evoked a concentration-dependent release of ³H-AA (Fig. 3) with an EC_{50} of 5 μM. Hill plot calculation by linear regression yields a slope of 0.989 ± 0.04 with a *r* squared = 0.996 (see inset, Fig. 3), indicating a noncooperative glutamate-evoked release of ³H-AA (Stryer, 1988). The efficacy of glutamate-evoked release of ³H-AA was maximal at a concentration of 100 μM and reached 189 ± 4% of basal (mean ± SEM, n = 133).

Pharmacological profile of glutamate receptors mediating the release of ³H-arachidonic acid

In agreement with previous studies, NMDA receptors are involved in the glutamate-evoked release of ³H-AA (Dumuis et al., 1988; Lazarewicz et al., 1988; Sanfeliu et al., 1990; Tapia-Arancibia et al., 1992). Indeed, 100 μM NMDA partially reproduced the effect of glutamate (Fig. 4). Addition of 100 μM D-ser-

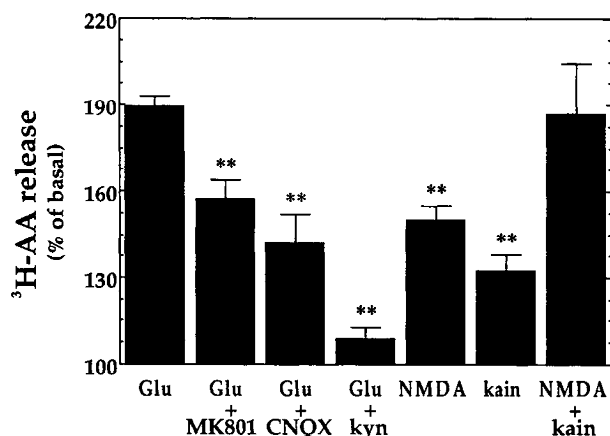

Figure 4. Effects of glutamatergic receptor agonists or antagonists on the release of ^{3}H-arachidonic acid. Cortical neurons were incubated for 15 min with the different glutamate receptor agonists: glutamate (*Glu*, 100 μM; n = 133), *NMDA* (100 μM; n = 48), and kainate (*kain*, 100 μM; n = 27). ^{3}H-AA release was determined as described in Materials and Methods, and results are expressed as percent of basal. Data are the mean ± SEM of n separate determinations and statistical analysis indicated: **, $p < 0.01$ significantly different from the glutamate-evoked ^{3}H-AA release (ANOVA followed by Dunnett's test). Antagonists of ionotropic glutamatergic receptors: *MK801* (1 μM; n = 18), *CNQX* (10 μM; n = 15) and kynurenate (*Kyn*, 1 mM; n = 12) were added both 15 min prior and during the 15 min incubation with 100 μM glutamate. Ionotropic antagonists alone did not significantly influence the basal release of ^{3}H-AA: MK801, 107 ± 5%, CNQX, 110 ± 10%, and kyn, 95 ± 7% of basal.

ine, an agonist of the glycine binding sites on NMDA receptors and a positive modulator of these receptors (Johnson and Ascher, 1987) did not significantly increase the NMDA-evoked release of ^{3}H-AA (139 ± 4% and 138 ± 5% of basal, without or with D-serine, respectively; both n = 6). Furthermore, the response evoked by 100 μM glutamate was partially inhibited by both competitive and noncompetitive antagonists of the NMDA receptors, that is, AP5 at 100 μM and MK801 at 1 μM (57 ± 7% and 63 ± 8% of the 100 μM glutamate response, respectively; Fig. 4). Other endogenous excitatory amino acids known to preferentially activate NMDA receptors were tested (Watkins et al., 1990). Thus, aspartate (100 μM), homocysteate (1 mM), and cysteate (1 mM) also evoked ^{3}H-AA release (151 ± 14%; 162 ± 6%, and 156 ± 10% of basal, respectively; for each agonist n = 9). The concentrations used were all maximally effective (not shown).

The involvement of AMPA/kainate receptors has also been proposed for the glutamate-evoked release of ^{3}H-AA (Patel et al., 1990). Here, neither the selective agonist of AMPA receptors, that is, AMPA at 100 μM nor the selective agonist of kainate receptors, that is, domoic acid at 1 μM (Watkins et al., 1990) significantly increased the release of ^{3}H-AA [106 ± 4% (n = 12) and 105 ± 4% (n = 6) of basal, respectively]. However, either kainate (100 μM) or AMPA (100 μM) in the presence of 100 μg/ml concanavalin A, an agent previously shown to prevent AMPA receptor desensitization (Huettner, 1990), were active (132 ± 6% and 147 ± 4% of basal, respectively; Fig. 4). This result suggests that the action of kainate may be mediated through nondesensitizing AMPA receptors. Accordingly, the stimulatory effect of glutamate was partially antagonized by either 10 μM CNQX or 10 μM DNQX (46 ± 11% and 35 ± 16% of the 100 μM glutamate response, respectively; Fig. 4).

Additivity of NMDA and AMPA/kainate receptor activation on the release of ^{3}H-arachidonic acid

An additive response between NMDA and AMPA/kainate receptor activation on the induced release of ^{3}H-AA has already been described on neuronal cultures originating from mouse striatal cells (Dumuis et al., 1990). At a maximally effective concentration, 100 μM NMDA in the presence of 100 μM kainate elicited an additive release of ^{3}H-AA (Fig. 4). Confirming the involvement of both NMDA and AMPA/kainate receptors in the glutamate response, a broad antagonist of ionotropic receptors, that is, kynurenate at 1 mM strongly antagonized the glutamate-evoked release of ^{3}H-AA (Fig. 4).

Noninvolvement of metabotropic receptors in the glutamate-evoked release of ^{3}H-arachidonic acid

Metabotropic glutamate receptors are present in 6 DIV cortical neurons, since 100 μM glutamate and 1 mM *t*-ACPD stimulated the formation of ^{3}H-IPs (200 ± 10% and 149 ± 10% of basal, n = 9). However, metabotropic glutamate receptors are not involved in the glutamate-evoked release of ^{3}H-AA. Indeed, *t*-ACPD at 1 mM did not significantly increase the basal release of ^{3}H-AA (110 ± 4% of basal, n = 12) and a competitive antagonist, that is, 1 mM AP3, failed to significantly decrease the glutamate-evoked release of ^{3}H-AA (92 ± 7% of the 100 μM glutamate response, n = 12). The combination of 100 μM AMPA and 1 mM *t*-ACPD did not significantly increase the basal release of ^{3}H-AA (112 ± 8% of basal, n = 9). Metabotropic glutamate receptors are linked to G-proteins (Nakanishi, 1992; Pin et al., 1994). When cortical neurons were incubated for 24 hr with 1 μg/ml pertussis toxin, both basal and glutamate-evoked release of ^{3}H-AA were unchanged, confirming that neither Gi nor Go proteins were involved in this response (not shown).

Extracellular pH modulates the glutamate-evoked release of ^{3}H-arachidonic acid

Because the activity of purified PLA_2 is pH sensitive (Moskowitz et al., 1983), we investigated the effect of extracellular pH (pH_o) changes on the glutamate-evoked release of ^{3}H-AA from cortical neurons in primary cultures. At this point, it is important to note that changes in pH_o are reflected within seconds by intracellular pH (pH_i) shifts in the same direction (Ou-yang et al., 1993).

The glutamate-evoked release of ^{3}H-arachidonic acid is pH_o sensitive

In these experiments, the incubation medium was buffered every 0.2 units between pH_o 7.2 and 7.8 (see Material and Methods). As shown in Figure 5, the release of ^{3}H-AA evoked by glutamate (100 μM) was enhanced as the extracellular medium was alkalinized. In particular, the glutamate response measured at pH_o 7.8 was 2.8-fold greater than that measured at 7.2. Using TLC, we verified that the glutamate-evoked release of ^{3}H-labeled product(s) observed at either 7.2 or 7.8 were exclusively accounted for by an increase in ^{3}H-AA release. In fact, the glutamate-evoked release of ^{3}H-AA seen by TLC was also 2.8-fold greater at pH_o 7.8 compared to 7.2. Thus, in two separate experiments, ^{3}H-AA–evoked release at pH_o 7.2 was 187 and 167 fmol/mg protein over basal, while at pH_o 7.8 it was 456 and 520 fmol/mg protein over basal, with a ^{3}H-AA basal release of 64 and 75 fmol/mg protein, respectively. Changes in pH_o rapidly modified the glutamate-evoked release of ^{3}H-AA. Indeed, a sig-

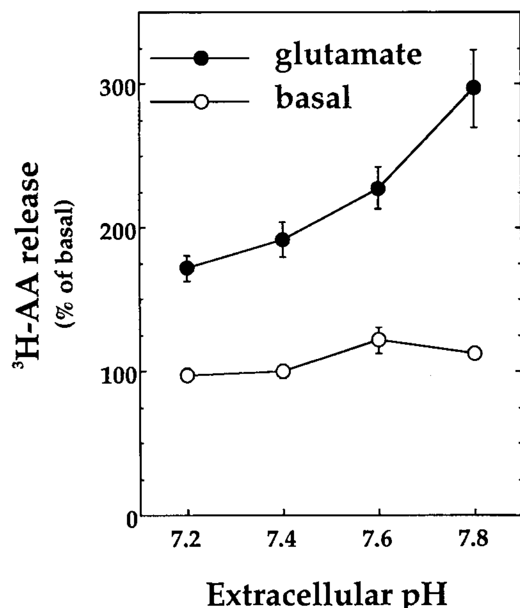

Figure 5. pH_o dependence of the glutamate-evoked release of ^{3}H-arachidonic acid. Cortical neurons were incubated for 15 min with 100 μM glutamate in a buffer at indicated pH. ^{3}H-AA release was determined as described in Materials and Methods. Results are the mean ± SEM of $n = 12$ separate determinations from four independent experiments.

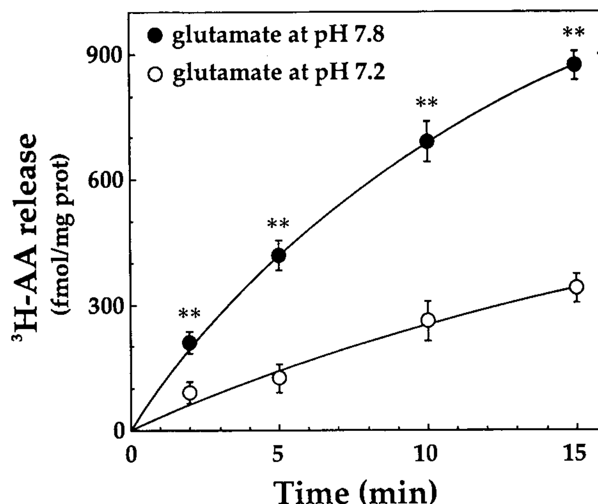

Figure 6. Kinetic of the pH_o-dependent glutamate-evoked release of ^{3}H-arachidonic acid. Cortical neurons were incubated for increasing periods with 100 μM glutamate in buffer at either pH_o 7.2 or 7.8 and ^{3}H-AA release was determined as described in Materials and Methods. Results are expressed as absolute increases in fmol per mg of protein above corresponding basal ^{3}H-AA release. Basal ^{3}H-AA release at each time point was 232 ± 24, 267 ± 15, 353 ± 22, and 492 ± 11 fmol/mg protein at, respectively, 2, 5, 10, and 15 min. Results are mean ± SEM of $n = 12$ separate determinations from four independent experiments. Statistical analysis: **, $p < 0.01$ significantly different from corresponding glutamate-evoked ^{3}H-AA release at pH_o 7.2 (unpaired Student *t* test).

nificant difference was observed 2 min after glutamate application at pH_o 7.2 or 7.8 (Fig. 6).

NMDA- and kainate-evoked release of ^{3}H-arachidonic acid are pH_o sensitive, while the increase in free intracellular Ca^{2+} is not

NMDA currents have been shown to be pH_o sensitive, whereas AMPA/kainate currents are not (Tang et al., 1990; Traynelis and Cull-Candy, 1990). Here, both the NMDA (100 μM) and the kainate (100 μM)-evoked release of ^{3}H-AA were increased by pH_o alkalinization (Fig. 7). This suggest that the pH-sensitive mechanism resulting in enhanced release of AA is independent of receptor activated currents. Interestingly, the NMDA- and kainate-evoked release of ^{3}H-AA measured at pH_o 7.8 were both ~ threefold greater than those observed at pH_o 7.2.

The activity of PLA_2 and the glutamate-evoked release of ^{3}H-AA are dependent on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Dumuis et al., 1988, 1993; Lazarewicz et al., 1990; Clark et al., 1991). Since activation of both NMDA and kainate receptors are known to induce $[Ca^{2+}]_i$ increases, a sensitivity of these $[Ca^{2+}]_i$ increases to pH could account for the pH sensitivity of ^{3}H-AA–evoked release. Hence, we measured these receptor-mediated $[Ca^{2+}]_i$ increases with the INDO-1 fluorescent dye at pH_o 7.2 and 7.8. The general pattern of NMDA- and kainate-induced $[Ca^{2+}]_i$ increases were similar when studied at both pH_o. In particular, application of either agonist at pH_o 7.2 or 7.8 revealed that initial $[Ca^{2+}]_i$ increases measured between basal and the beginning of $[Ca^{2+}]_i$ plateau levels, that is, 10 sec, were unchanged. $[Ca^{2+}]_i$ increase evoked by NMDA studied in 70 cortical neurons was 71 ± 8 and 86 ± 7 nM/sec, at pH_o 7.2 and 7.8, respectively. Furthermore, kainate-induced $[Ca^{2+}]_i$ increases in 67 cortical neurons was 28 ± 3 and 31 ± 4 nM/sec, at pH_o 7.2 and 7.8; respectively. No significant difference was found when NMDA- and kainate-induced $[Ca^{2+}]_i$ increases were compared at each pH_o (two-tailed unpaired Student *t* test; $p > 0.05$). Table 1 shows that a similar $[Ca^{2+}]_i$ plateau value was reached at either pH_o 7.2 or 7.8 after NMDA or kainate application.

Intracellular pH modulates the glutamate-evoked release of ^{3}H-arachidonic acid

It has been shown that pH_i can be changed by directly affecting H^+ equivalents exchange (Ou-yang et al., 1993). In hippocampal neurons, it has been shown that the resting pH_i is 0.2–0.3 higher when cells are maintained in a HCO_3-buffered solution com-

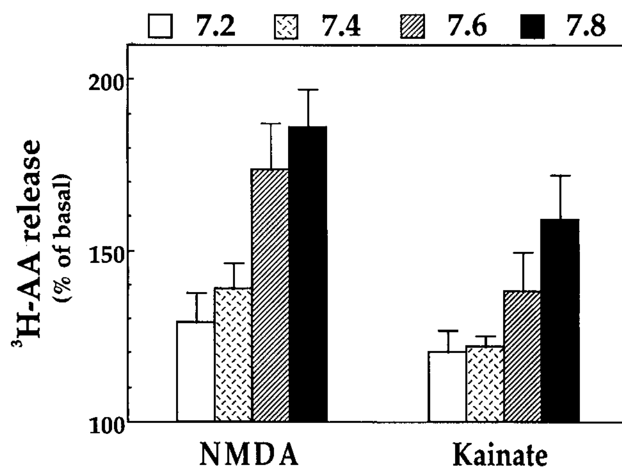

Figure 7. pH_o Dependence of both NMDA- and the kainate-evoked release of ^{3}H-arachidonic acid. Cortical neurons were incubated for 15 min with NMDA (100 μM) or kainate (100 μM) in buffer at indicated pH_o. ^{3}H-AA release was determined as described in Materials and Methods. Results are mean ± SEM of $n = 12$ separate determinations from four independent experiments.

Table 1. Insensitivity to pHo of NMDA- and kainate-induced $[Ca^{2+}]_i$ elevations

pH	$[Ca^{2+}]_i$ (nM) Basal	NMDA	Kainate
7.2	57 ± 3	673 ± 68	419 ± 53
7.8	43 ± 2	745 ± 54	397 ± 39

Elevation in $[Ca^{2+}]_i$ was measured in INDO-1–loaded cortical neurons exposed for a period of 2 min either to 100 μM NMDA + 100 μM D-serine or to 100 μM kainate. Incubation medium lacking Mg^{2+} was buffered at the indicated pH. Data represent changes in $[Ca^{2+}]_i$ calculated as described in Materials and Methods, corresponding to n individual neurons subjected to identical treatments and chosen at random from three independent coverslips. n = 66 and 71 for NMDA + D-serine at pH 7.2 and 7.8, respectively; n = 58 and 75 for kainate at pH 7.2 and 7.8, respectively. No significant difference was found when NMDA and kainate responses were compared at each pH (two-tailed unpaired Student t test, $p > 0.05$).

pared to a nominally HCO_3-free solution (Raley-Susman et al., 1991; Schwiening and Boron, 1994). In line with these observations, glutamate (100 μM) evoked a significantly smaller release of ^{3}H-AA when $NaHCO_3$ was removed from the buffered solution (see Materials and Methods) (190 ± 8% and 171 ± 6% of basal, with and without 3.6 mM $NaHCO_3$, respectively; n = 6; $p < 0.05$ with two-tailed unpaired Student t test). To further confirm this observation, we incubated cortical neurons in a buffer containing 30 mM NH_4Cl, a treatment that is known to alkalinize pH_i (Raley-Susman et al., 1991; Schwiening and Boron, 1994). Following this treatment, the glutamate response was also enhanced (Table 2).

Two major types of specific antiport are involved in the proton homeostasis of cells, the Na^+/H^+ and the Cl^-/HCO_3^- carrier. Thus, inhibition of both activities results in intracellular acidosis (Hartley and Dubinsky, 1993; Ou-yang et al., 1993). Incubation of cells with glutamate (100 μM) in the presence of Na^+/H^+ exchange inhibitors, that is, amiloride at 1 mM or harmaline at 100 μM, significantly inhibited the glutamate-evoked ^{3}H-AA release (Table 2). Likewise, inhibition of Cl^-/HCO_3^- by 100 μM DIDS also significantly decreased the glutamate-evoked ^{3}H-AA release (Table 2).

pH Sensitivity of membrane-bound Ca^{2+}-dependent PLA_2 activities

PLA_2 activities are present both in the cytoplasmic compartment ($cPLA_2$) and bound to the plasma membrane ($mPLA_2$). In cortical synaptosomes, Ca^{2+}-independent and Ca^{2+}-dependent PLA_2 activity for each compartment has been described (Piomelli and Greengard, 1991). We therefore studied the effect of pH on these four PLA_2 activities.

pH sensitivity of membrane-bound PLA_2 activity. When membranes were incubated at pH 7.2 in the presence of the fluorogenic PLA_2 substrate PPC, a basal Ca^{2+}-independent $mPLA_2$ activity was measured (4.7 nmol/mg of protein/sec). Increasing the pH of incubation medium from 7.2 to 7.8 did not change this basal Ca^{2+}-independent $mPLA_2$ activity, which was measured over a 2 min period (see Materials and Methods and Fig. 8*A*, before Ca^{2+} addition).

Addition of 2 mM Ca^{2+} revealed a Ca^{2+}-dependent $mPLA_2$ activity. Ca^{2+} induced a transient acceleration of the $mPLA_2$ activity that reached a maximum at 20 sec, returning to the initial basal activity after another 20 sec (Fig. 8*A*). Ca^{2+} addition not only induced an initial acceleration of the Ca^{2+}-dependent $mPLA_2$, but it was also followed by a transient inhibition of the

Table 2. Effect of pH-regulating carrier inhibitors on the glutamate-evoked release of ^{3}H-arachidonic acid

Agents added	mM	^{3}H-AA release (% of basal release) Basal	+ Glutamate
None		100 ± 2	188 ± 8
NH_4Cl	30	132 ± 7	231 ± 7**
Harmaline	0.1	86 ± 13	101 ± 9**
Amiloride	1	100 ± 5	116 ± 9**
DIDS	0.1	106 ± 5	144 ± 10**

Cortical neurons were incubated for 15 min with different pH-regulating carriers inhibitors. Agents were added at the same time as glutamate. ^{3}H-AA release was determined as described in Materials and Methods. Results are expressed as percentage of basal ^{3}H-AA release. Results for each treatment are the mean ± SEM of n = 12 separate determinations from four independent experiments.

** $p < 0.01$ compared to glutamate-evoked ^{3}H-AA release (ANOVA followed by Dunnett's test).

$mPLA_2$ activity (Fig. 8*A*). This inhibitory process occurred in two phases: first a strong inhibition occurred for approximately 60 sec, followed by a weaker inhibition that was maintained for at least 200 sec. Together, the two phases of the inhibitory process were always lower than the basal $mPLA_2$ activity (Fig. 8*A*).

Ca^{2+}-dependent $mPLA_2$ activity measured at 20 sec after addition of Ca^{2+} was increased as the incubation pH was changed every 0.2 pH units from 7.2 to 7.8 (Fig. 8*B*). Interestingly, the Ca^{2+}-dependent $mPLA_2$ activity measured at 20 sec in the incubation medium buffered at 7.8 was 3.4-fold greater than the response measured at 7.2. In addition, the inhibitory phase that follows Ca^{2+}-evoked $mPLA_2$ activation was less pronounced at 7.8 than at 7.2 (Fig. 8*A*).

pH insensitivity of cytosolic PLA_2 activity. When cytoplasmic homogenates were incubated at pH 7.2 in the presence of fluo-

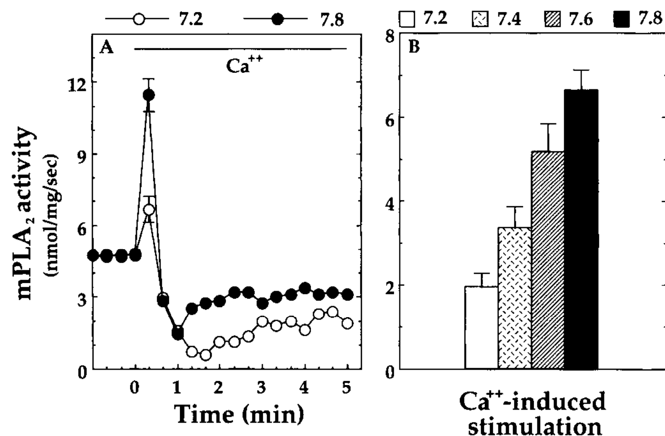

Figure 8. pH Sensitivity of membrane-bound Ca^{2+}-dependent PLA_2 activity. *A*, Membranes were prepared from three independent neuronal cultures and $mPLA_2$ activity was measured in a 50 mM Tris-HCl medium buffered at indicated pH, as described in Materials and Methods. Data are the result of n = 4 independent measurements of $mPLA_2$ activity expressed in nmol of pyrenedecanoate released/mg of protein/sec. For graphic clarity, only 1 min of the basal, Ca^{2+}-independent, $mPLA_2$ activity are represented, and error bars are indicated only the time point at 20 sec after Ca^{2+} addition. At other time-point, SEM were < 10%. *B*, The Ca^{2+}-induced stimulation of $mPLA_2$ activity expressed as (nmol of pyrenedecanoate released over basal/mg of protein/sec) measured between 5 and 20 sec after application of Ca^{2+}. $mPLA_2$ activity was measured as described in *A*.

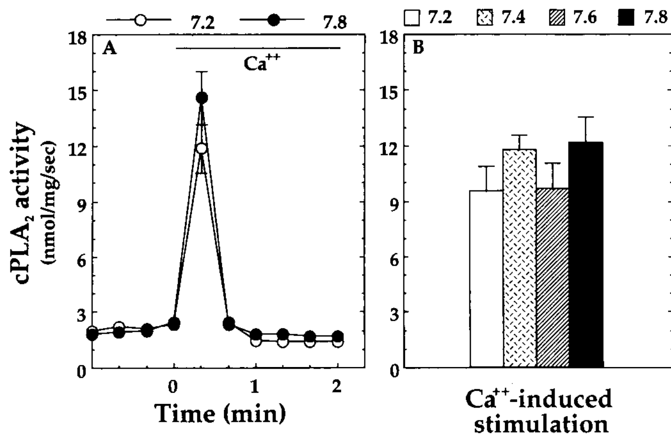

Figure 9. pH Insensitivity of cytoplasmic PLA_2 activity. *A*, Cytoplasm were prepared from three independent neuronal cultures, and $cPLA_2$ activity was measured in a 50 mM Tris-HCl medium buffered at indicated pH as described in Materials and Methods. Data are the result of $n = 4$ independent measurements of $cPLA_2$ activity expressed in nmol of pyrenedecanoate released/mg of protein/sec. For graphic clarity, only 1 min of the basal, Ca^{2+}-independent, $cPLA_2$ activity are represented, and error bars are indicated only the time point at 20 sec after Ca^{2+} addition. At other time point, SEM were $< 10\%$. *B*, The Ca^{2+}-induced stimulation of $cPLA_2$ activity expressed as (nmol of pyrenedecanoate released over basal/mg of protein/sec) measured between 5 and 20 sec after application of Ca^{2+}. $cPLA_2$ activity was measured as described in *A*.

rogenic PLA_2 substrate, a basal Ca^{2+}-independent $cPLA_2$ activity was measured (2.3 nmol/mg of protein/sec). Increasing the pH of incubation medium from 7.2 to 7.8 did not change this basal Ca^{2+}-independent $cPLA_2$ activity, which was measured over a 2 min period (see Materials and Methods and Fig. 9*A*, before Ca^{2+} addition).

Addition of 2 mM Ca^{2+} revealed a Ca^{2+}-dependent $cPLA_2$ activity. Ca^{2+} induced a transient acceleration of the $cPLA_2$ activity with a maximum at 20 sec, which then returned to the initial basal activity after another 20 sec (Fig. 9*A*). In contrast to $mPLA_2$, this activity remained stable (Fig. 9*A*). As pH of the incubation medium was increased from 7.2 to 7.8, the Ca^{2+}-dependent $cPLA_2$ measured at 20 sec was not significantly affected (Fig. 9*B*).

Discussion

Characterization of cortical neurons in primary culture

In general, neurons in primary cultures are grown in a medium containing varying degrees of fetal serum; alternatively, the culture dishes are coated with serum. These procedures are known to enhance cell viability, but they also result in the presence of a certain percentage of either glial cells or at least glial precursors (DiPorzio et al., 1980). Since astrocytes influence neuronal functions and, in particular, glutamate responses (Bouvier et al., 1992; Glowinski et al., 1994; Parpura et al., 1994), it was important to examine the effect of glutamate on neuronal cultures grown in conditions that prevent glial proliferation. We therefore used cortical neurons cultured in the total absence of serum. Under these conditions, cortical neurons reached a maximal viability between 6 and 7 DIV (Fig. 1), similar to what has been described for mesencephalic neurons cultured under the same conditions (DiPorzio et al., 1980).

Virtually all cells were labeled by antibodies directed against neuronal markers (Fig. 2). In contrast, GFAP immunoreactivity was still absent at 11 DIV, a stage where astrocytes in culture have been shown to express this specific marker (Trimmer et al., 1982). These results strongly suggest that astrocytes were absent from the neuronal cultures used in this study.

It has been previously shown that cortical cultures exposed to glutamate results in a neurotoxicity, which is inversely related to the number of astrocytes present in the culture (Rosenberg et al., 1992); hence, neurotoxicity due to glutamate is higher in astrocyte-poor cultures. Furthermore, the toxicity of glutamate also depends on the age of neurons in primary culture; thus, neurons cocultured with astrocytes are sensitive to glutamate toxicity only after 13 DIV (Choi et al., 1987). Since, the culture conditions used in the present study yield cortical neurons in the absence of glial cells (see Materials and Methods) and reach their maximal viability at 6 DIV (Fig. 1), it was important to determine the potential neurotoxicity of glutamate under the experimental conditions used. As described in the Materials and Methods section, application of glutamate at 100 μM for 15 min did not affect neuronal viability as indicated by Trypan blue exclusion.

Excitatory amino acids induce the release of ^{3}H-arachidonic acid from cortical neurons in primary culture

We showed that glutamate stimulates the release of AA in mouse cerebral cortical neurons in glia-free primary cultures. It has been recognized that the physiological actions of glutamate are mediated by a family of receptors classified in three main classes: NMDA, AMPA/kainate, and metabotropic receptors (for review, see Nakanishi, 1992). Recent molecular and pharmacological studies have shown that several receptor subtypes exist for each of these classes.

The receptors mediating the glutamate-evoked release of AA in the present study are of the NMDA and AMPA classes. First, NMDA reproduces 56% of the glutamate response and the glutamate-evoked release of AA is reduced by half, both by competitive and noncompetitive antagonists of the NMDA receptors (Fig. 4; Results). Furthermore, aspartate, homocysteate, and cysteate also evoked approximately 50% of the glutamate maximal response. These excitatory amino acids are known to preferentially activate NMDA receptors (Watkins et al., 1990), further confirming the involvement of NMDA receptors in part of the glutamate-evoked release of AA. Interestingly, a physiological role as endogenous excitatory neurotransmitters has been documented for aspartate, homocysteate, and cysteate (Watkins et al., 1990; Do et al., 1992). In line with results reported here, aspartate has also been shown to stimulate AA release in cerebellar granule cells with an efficacy that is 50% of that of glutamate (Lazarewicz et al., 1988).

Second, when AMPA receptor desensitization is prevented by concanavalin A, AMPA reproduces 53% of the glutamate response. Similarly, kainate exerts a stimulatory effect that reaches 36% of that observed with glutamate. The modest response induced by kainate could be due to its action on AMPA receptors, which are known to desensitize marginally after activation with this agonist (Barnard and Henley, 1990). The direct involvement of kainate receptors is unlikely, since the specific agonist of kainate receptors, that is, domoic acid (Watkins et al., 1990) was ineffective. Finally, the glutamate-evoked release of AA was reduced by approximately 50% by competitive antagonists of the AMPA/kainate receptors (Fig. 4; Results).

Third, metabotropic receptors do not appear to be involved in the glutamate-evoked release of AA, since neither an agonist

mimicked nor an antagonist inhibited the effect of glutamate. Contrary to what has been described in 14 DIV striatal neurons in which the combined stimulation of AMPA and metabotropic receptors induces the release of AA (Dumuis et al., 1990, 1993), in 6 DIV cortical neurons, metabotropic receptor activation has no effect on the AMPA response.

Finally, an additive stimulation of AA release occurs when NMDA and AMPA receptors are coactivated, which then totally reproduces the glutamate response. It is generally accepted that non-NMDA receptors are involved in low frequency excitatory amino acid-mediated synaptic transmission, whereas NMDA receptors become functionally operational mainly after further depolarization of the membrane (Herron et al., 1986) by removing the Mg^{2+} block of the channel (Mayer et al., 1984; Nowak et al., 1984). In view of its pharmacological profile, it is clear that activation of both receptor types is needed to achieve the full expression of the glutamate-evoked AA release.

pH Sensitivity of the glutamate-evoked release of ^{3}H-arachidonic acid

A dependence on extracellular pH (pH_o) has been previously reported for NMDA currents, while AMPA/kainate currents remained unaffected (Tang et al., 1990; Traynelis and Cull-Candy, 1990). In contrast, results reported here show that in addition to the NMDA-evoked release of AA, the effect of kainate is also enhanced in alkaline buffer (Fig. 7). These observations suggest that pH_o might not modify the glutamate-evoked release of AA by directly affecting the conductance of a given receptor-channel class, but rather by modulating an intracellular step further downstream in the pathway that leads to AA liberation.

It is well known that a crucial step involved in the glutamate-evoked release of AA is the Ca^{2+} influx induced by glutamatergic receptor activation (Dumuis et al., 1988, 1993; Lazarewicz et al., 1990). If both NMDA- and kainate-induced $[Ca^{2+}]_i$ increase were pH sensitive, this could be a possible mechanism responsible for the pH sensitivity of AA release induced by both agonists. However, this hypothesis can be ruled out, since both NMDA- and kainate-induced increases in $[Ca^{2+}]_i$ were not significantly affected by pH_o (Table 1; Results). These observations are in agreement with a recent report indicating the pH insensitivity of the NMDA-induced Ca^{2+} influx in rat hippocampal neurons (Irwin et al., 1994).

PLA_2 activity has been shown to be directly dependent on H^+ concentration (Moskowitz et al., 1983). Indeed, we observed that pH_o rapidly increases the glutamate-evoked release of AA, since a significant difference was observed within 2 min glutamate application in a medium buffered at 7.8 versus 7.2. These results are in agreement with the rapid switch in pH_i that occurs within seconds after changes in pH_o (Ou-yang et al., 1993). Furthermore, we demonstrated that manipulations known to directly affect pH_i also modified the glutamate-induced release of AA. Thus, it is well documented that when NH_4Cl (in the 20–40 mM range) is applied to the incubation medium, a biphasic variation in pH_i occurs (Raley-Susman et al., 1991): initially, neuronal pH_i increases (presumably due to the influx of the membrane-permeable base NH_3; Schwiening and Boron, 1994), then an acidification rebound occurs when NH_4Cl is removed (due to the activation of pH regulatory countertransport mechanisms). As shown in Table 2, when neuronal cultures are exposed to NH_4Cl, the glutamate-evoked release of ^{3}H-AA is enhanced at a time point when the pH_i is alkaline.

Inhibition of both Na^+/H^+ and the Cl^-/HCO_3^- exchange have been shown to induce a pH_i acidification (L'Allemain et al., 1985; Raley-Susman et al., 1991). Indeed, when cortical neurons are treated with both harmaline or amiloride, two inhibitors of the Na^+/H^+ exchange, the glutamate-evoked release of AA is strongly inhibited (Table 2). Interestingly, Na^+/H^+ exchange in hippocampal neurons is sensitive to 100 μM harmaline and insensitive to 1 mM amioride (Raley-Susman et al., 1991); whereas Cl^-/HCO_3^- exchange is inhibited by 100 μM DIDS (Schwiening and Boron, 1994). As shown in Table 2, DIDS also inhibited the glutamate-evoked release of AA in cortical neurons.

Taken together, this set of results demonstrates that pH_o regulates the glutamate-evoked release of ^{3}H-AA from cortical neurons, without significantly affecting the glutamate-evoked increase in $[Ca^{2+}]_i$, but rather by directly affecting pH_i.

pH Sensitivity of Ca^{2+}-dependent membrane-bound PLA_2 activity

Data reported in this article clearly indicate that cortical neurons in primary cultures possess four different PLA_2 activities, that is, Ca^{2+}-independent or Ca^{2+}-dependent membrane-bound PLA_2 ($mPLA_2$) and cytoplasmic PLA_2 ($cPLA_2$) activities. These results are in agreement with those previously obtained in rat cortical synaptosomes (Piomelli and Greengard, 1991). Various molecular mechanisms underlying the Ca^{2+}-dependent activation of either $cPLA_2$ or $mPLA_2$ activity could be considered. For example, Ca^{2+} activated proteins, such as protein kinase C (Lin et al., 1992) and Ca^{2+}/calmodulin kinase II (Piomelli and Greengard, 1991) have been shown to regulate PLA_2 activity. Alternatively, Ca^{2+} may directly activate PLA_2, since this ion has been shown to directly bind $cPLA_2$ (Clark et al., 1991).

In the present study, we studied the pH sensitivity of the four different PLA_2 activities. We observed that only the Ca^{2+}-dependent $mPLA_2$ activity is modulated by pH (Fig. 8*A,B*), while the Ca^{2+}-independent $mPLA_2$ activity is not. Furthermore, both Ca^{2+}-independent and Ca^{2+}-dependent $cPLA_2$ activities are pH insensitive (Fig. 9*A,B*). Thus, considering the fact that pH_i alkalinization enhances $mPLA_2$ activity, it can be postulated that as pH_i changes to alkaline values, a Ca^{2+} influx due to glutamatergic receptor activation will then preferentially stimulate $mPLA_2$ activity. Indeed, the glutamate-evoked release of AA requires Ca^{2+} influx (Dumuis et al., 1988, 1993; Lazarewicz et al., 1990) ruling out the Ca^{2+}-independent PLA_2 activities. In addition, stimulation of AA release by activation of NMDA and AMPA receptors are pH sensitive (Fig. 7), with a pH profile similar to that pH profile of Ca^{2+}-dependent $mPLA_2$ activity. It should be emphasized that, in the experimental condition used in this study, that is, measuring PLA_2 activity with PPC, it is not possible to asses the translocation of $cPLA_2$, since cytoplasm and plasma membranes were analyzed separately. Nevertheless, it cannot be exclude that $cPLA_2$ or related enzyme could also be involved once it has translocated to the membrane of intact neurons.

The exact molecular mechanism(s) involved in the pH sensitivity of $mPLA_2$ activity remain(s) to be clarified. Reports on human U937 cells $cPLA_2$ have shown that this 85.2 kd protein contains a Ca^{2+}-dependent domain (Clark et al., 1991). Whether H^+ concentration directly influences this domain or whether pH alters the properties of cofactors necessary for PLA_2 activity is not known. In keeping with previous observations in rat cortical synaptosomes (Piomelli and Greengard, 1991), the Ca^{2+}-dependent activity of the $mPLA_2$ was modulated in a biphasic manner by Ca^{2+}. Thus, after a rapid (20 sec) increase in activity follow-

ing Ca^{2+} addition, $mPLA_2$ activity decreased rapidly and was actually inhibited to levels lower than basal (Fig. 8*A*). This inhibition has been interpreted as a negative feed-back mechanism exerted by Ca^{2+} acting through a Ca^{2+}/calmodulin kinase II (Piomelli and Greengard, 1991). As shown in Figure 8, alkalinization enhances the initial Ca^{2+}-dependent activation of $mPLA_2$ (Fig. 8*B*) and decreases the Ca^{2+}-mediated feed-back inhibition (Fig. 8*A*); thus, the net result of alkalinization is a long-lasting increase in $mPLA_2$ activity.

Following electrically induced depolarization, a 1–2 min pH_o alkalinization is observed (Kraig et al., 1983; Chesler and Chan, 1988). It has been proposed that pH_o changes are due to the exchange of H^+ equivalents principally between the interstitial space and surrounding glial cells (Chesler, 1990; Ransom, 1992). In line with this hypothesis, Bouvier et al. demonstrated that glutamate uptake into glial cells is associated with the countertransport of OH^- into the extracellular medium (Bouvier et al., 1992). Thus, the extracellular alkalinization could result from this neuronal–glial interaction due to glutamate reuptake into astrocytes. Changes in pH_o lasting for several minutes could modulate neuronal responses by affecting the properties of membrane proteins such as receptors that possess a domain facing the extracellular space. Indeed, NMDA currents determined in the whole-cell mode are pH_o sensitive (Tang et al., 1990; Traynelis and Cull-Candy, 1990). Accordingly, epileptiform activity mediated by NMDA receptors is enhanced by pH_o alkalinization and dampened by pH_o acidification (Aram and Lodge, 1987).

Intracellular pH is also altered during glutamate neurotransmission. First, changes in pH_o influence pH_i within seconds by shifting its values in the same direction (Ou-yang et al., 1993). Second, it should be emphasized that activation of NMDA receptors per se has been described to induce long-term changes in intracellular H^+ homeostasis. This mechanism provides an additional level of complexity to the modulation of intracellular H^+ concentration. Indeed, activation of NMDA receptors first induces a transient pH_i acidification (Endres et al., 1986; Irwin et al., 1994) followed by pH_i alkalinization that can persist for several hours (Hartley and Dubinsky, 1993). This long-lasting alkalinization will enhance the efficacy of glutamate in evoking AA release upon subsequent activation, thus providing a long-lasting facilitation of specific signal transduction pathway at glutamatergic synapse.

Certain components of long-term potentiation (LTP) appear to involve AA formation (Williams et al., 1989; Drapeau et al., 1990). AA can, in fact, regulate the efficacy of glutamatergic neurotransmission by increasing NMDA currents (Miller et al., 1992) or glutamate concentration in the synaptic cleft (Yu et al., 1986; Barbour et al., 1989; Herrero et al., 1992; Volterra et al., 1992). Since glutamate evokes long-lasting pH_i alkalinizations (Hartley and Dubinsky, 1993), which, in turn, enhances the capacity of glutamate to evoke AA release, it may be suggested from the foregoing that the pH sensitivity of glutamate-evoked AA release may contribute to the long-lasting increase in synaptic efficacy characterized for LTP.

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