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Extracellular protons differentially potentiate the responses of native AMPA receptor subtypes regulating neurotransmitter release

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1 The effects of pH changes on the basal and evoked release of [^{3}H]noradrenaline ([^{3}H]NA) and [^{3}H]5-hydrohytryptamine ([^{3}H]5-HT) from hippocampal synaptosomes and of [^{3}H]dopamine ([^{3}H]DA) and [^{3}H]acetylcholine ([^{3}H]ACh) from striatal and cortical synaptosomes were investigated in rat brain.

2 Changing pH between 6.4 and 8.0 did not affect the spontaneous release of the four [^{3}H]neurotransmitters; alkalinization to pH 8.8 significantly enhanced release. Acidification to pH 6.4 augmented the AMPA-evoked overflows of [^{3}H]NA, [^{3}H]5-HT and [^{3}H]DA, but not that of [^{3}H]ACh. In contrast, lowering pH to 6.4 decreased the K^+-evoked overflows of [^{3}H]NA, [^{3}H]5-HT, [^{3}H]DA and [^{3}H]ACh.

3 AMPA released transmitters in a Ca^{2+}-dependent, exocytotic manner since its effects, at pH 7.4 or 6.4, were abolished by omitting external Ca^{2+} or by depleting vesicular transmitter stores with bafilomycin A1. AMPA did not evoke carrier-mediated release because the uptake blockers nisoxetine, 6-nitroquipazine, GBR12909 and hemicholinium-3 could not inhibit the AMPA-induced release of [^{3}H]NA, [^{3}H]5-HT, [^{3}H]DA and [^{3}H]ACh.

4 Extraterminal acidification to pH 6.4 prevented the potentiating effect of cyclothiazide on the AMPA-evoked release of [^{3}H]NA, [^{3}H]DA and [^{3}H]5-HT, whereas the proton-insensitive AMPA-evoked release of [^{3}H]ACh, previously found to be cyclothiazide-insensitive at pH 7.4 was cyclothiazide-resistant also at pH 6.4.

5 To conclude, the cyclothiazide-sensitive AMPA receptors mediating release of NA, 5-HT and DA, but not the cyclothiazide-insensitive AMPA receptors mediating the release of ACh, become more responsive when external pH is lowered to pathophysiologically relevant values. The results with cyclothiazide suggest that H^+ ions may prevent desensitization of some AMPA receptor subtypes.
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Abbreviations: ACh, acetylcholine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; DA, dopamine; NA, noradrenaline; 5-HT, serotonin

Introduction

AMPA/kainate receptors mediate fast excitatory synaptic transmission exerting relevant roles in development, neuroplasticity and excitotoxicity (for reviews see Hollmann & Heinemann, 1994; Bettler & Mulle, 1995; Bleakman & Lodge, 1998, Dingledine *et al*., 1999). Evidence has been provided that rat hippocampal noradrenergic and serotonergic terminals, as well as striatal dopaminergic and cortical cholinergic nerve endings, are endowed with AMPA/kainate receptors whose activation causes Ca^{2+}-dependent exocytotic-like neurotransmitter release. The pharmacological characterization of these receptors, obtained with a number of receptor antagonists, suggests that they all belong to the AMPA-preferring type, but appear to represent four pharmacologically distinct subtypes (Ghersi *et al*., 2003). It is well known that AMPA receptors undergo desensitization, which can be prevented by drugs like cyclothiazide (Partin *et al*., 1993; Bettler & Mulle, 1995; Bleakman & Lodge, 1998); the existence of endogenous factors mimicked by these cyclothiazide-like compounds is unknown.

Glutamate receptors of the NMDA type exhibit decreased function when extracellular pH is lowered to pathophysiologically relevant values (Traynelis & Cull-Candy, 1990; Traynelis *et al*., 1995; Pittaluga *et al*., 2001). In contrast, the relationships between acidification and function of AMPA receptors are controversial. These receptors have been reported to be unaffected by protons at pH 6.5 (see Traynelis, 1998). On the other hand, at pH values lower than 6.5, which are of more biochemical than pathophysiological interest, the responses to AMPA were found to be inhibited (Christensen & Hida, 1990; Traynelis & Cull-Candy, 1991; Traynelis *et al*., 1995; Lei *et al*., 2001). Recent results, obtained in primary cell cultures from murine brain, show that

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extracellular acidification could inhibit AMPA receptor-mediated responses (Ihle & Patneau, 2000), but potentiated AMPA receptor-mediated excitotoxicity (McDonald *et al*., 1998).

The aim of the present work was to investigate whether the function of native AMPA receptors mediating enhancement of noradrenaline (NA), dopamine (DA), 5-HT and ACh release in nerve endings isolated from adult rat brain is sensitive to extracellular pH changes. We here find that protons potentiate the release evoked by AMPA from the vesicular pool of NA, DA and 5-HT, leaving unaffected the AMPA-evoked release of ACh. Moreover, our results show that H^+ ions strengthen the function of release-regulating AMPA receptors previously found to be cyclothiazide-sensitive, but not that of receptors found to be insensitive to the drug (Pittaluga *et al*., 1997).

Methods

Animals

Adult male rats (Sprague–Dawley, 200–250 g) were housed at constant temperature (22 ± 1°C) and relative humidity (50%) under a regular light–dark schedule (light 07:00–19:00). Food and water were freely available. The experimental procedures were approved by the Ethical Committee of the Pharmacology and Toxicology Section, Department of Experimental Medicine, in accordance with the European legislation (European Communities Council Directive of 24 November 1986, 86/609/EEC).

Preparation of synaptosomes

Rats were killed by decapitation, the brains were rapidly removed and cortices, striata and hippocampi were dissected out. Crude synaptosomes were prepared according to Raiteri *et al*. (1984). Briefly, tissues were homogenized in 40 volumes of 0.32 M sucrose, buffered to pH 7.4 with phosphate (final concentration 0.01 M). The homogenate was centrifuged at $1000 \times g$ for 5 min, to remove nuclei and cellular debris, and crude synaptosomes were isolated from the supernatant by centrifugation at $12{,}000 \times g$ for 20 min. The synaptosomal pellet was then resuspended in a physiological medium having the following composition (mM): NaCl, 136; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1; $NaHCO_3$, 5; glucose, 10; HEPES, 5; pH 7.2–7.4. Hippocampal synaptosomes were incubated 15 min at 37°C with [^{3}H]NA (final concentration 30 nM) or with [^{3}H]5-HT (final concentration 80 nM), in the presence of 0.1 μM 6-nitroquipazine or 0.1 μM nisoxetine to avoid false labelling of serotonergic and noradrenergic terminals, respectively. Striatal synaptosomes were incubated 15 min at 37°C with [^{3}H]DA (final concentration 23 nM), while cortical synaptosomes were labelled with [^{3}H]choline (final concentration 30 nM). When studying the vesicular origin of the AMPA-evoked neurotransmitter release, synaptosomes were preincubated with the vesicular ATPase blocker bafilomycin A1 (Bowman *et al*., 1988), final concentration 0.1 M, for 15 min at 37°C and then incubation was carried out with the appropriate radioactive tracer as previously described.

Release experiments

Identical portions of the synaptosomal suspension were layered on microporous filters at the bottom of parallel superfusion chambers maintained at 37°C (see, for details, Raiteri & Raiteri, 2000) and superfused at 0.5 ml min^{-1} with standard physiological solution for 36 min; a 90 s period of stimulus (different pH, 15 mM KCl or 100 μM AMPA, in the presence or absence of cyclothiazide) was then applied at $t = 39$ min. The pH of the superfusion medium (standard medium, pH 7.4) was adjusted to the desired value (6.4, 6.8, 8.0 or 8.8) with HCl or NaOH.

In a series of experiments carried out to study the Ca^{2+}-dependency of the AMPA-evoked release, at different pH, the superfusion medium was replaced, at $t = 20$ min, with a medium from which Ca^{2+} ions were omitted and to which 0.5 mM EGTA was added. When testing the effects of transporter blockers, 0.1 μM nisoxetine (experiments of [^{3}H]NA release), 0.1 μM 6-nitroquipazine (experiments of [^{3}H]5-HT release), 0.1 μM GBR12909 (experiments of [^{3}H]DA release) or 10 μM hemicholinium-3 (experiments of [^{3}H]ACh release) was added 8 min before AMPA and maintained till the end of superfusion.

Fraction collection was started at $t = 36$ min according to the following scheme: two 3-min samples (basal release) before (min 36–39) and after (min 45–48) one 6-min sample (evoked release, min 39–45) containing the [^{3}H]neurotransmitters released by pH changes, high-K^+ or AMPA. AMPA (alone or in the presence of 10 μM cyclothiazide) was added concomitantly with the pH stimulus, as indicated. Fractions collected and superfused synaptosomes were then counted for radioactivity.

Calculations and statistics

The tritium present in the superfusate samples and that remaining in the synaptosomes at the end of superfusion were expressed as percentages of the total tritium content at the start of the respective collection period (fractional rate $\times$ 100). The evoked [^{3}H]neurotransmitter release was expressed as induced overflow and was calculated by subtracting the neurotransmitter content into the first and the third fractions collected (basal release) from that in the 6 min-fraction collected during and after the depolarization pulse (evoked release).

Analysis of variance was performed by ANOVA followed by Dunnett's test or Student's *t*-test, as appropriate. Data were considered significant for $P < 0.05$ at least.

Chemicals

1-[7,8-^{3}H]NA (specific activity 39 Ci mmol^{-1}), [7,8-^{3}H]dopamine (specific activity 43 Ci mmol^{-1}), [methyl-^{3}H]choline (specific activity 83 Ci mmol^{-1}) were from Amersham Radiochemical Center (Buckinghamshire, U.K.); 5[1,2-^{3}H(*N*)]hydroxytryptamine creatinine sulfate (specific activity 38 Ci mmol^{-1}) from NEN, DuPont products (Boston, MA, U.S.A.). Nisoxetine hydrochloride, bafilomycin A1, 6-nitroquipazine maleate and AMPA from Tocris-Cookson (Bristol, U.K.) while hemicholinium-3 was from Aldrich (Milwaukee, WI, U.S.A.). The following compounds were kindly gifted:

cyclothiazide from Eli Lilly (Indianapolis, IN, U.S.A.) and GBR12909 from Gist Brocades, the Netherlands.

Results

Effects of pH changes on the spontaneous release of [^{3}H]neurotransmitters

Figure 1 shows the spontaneous [^{3}H]neurotransmitter release from synaptosomes exposed in superfusion (for 90 s) to media of different pH (6.4–8.8). At pH 7.4, taken as physiological pH value, the release of tritium amounted to 0.12 ± 0.05, 0.02 ± 0.06, 0.02 ± 0.04 and 0.13 ± 0.04% from synaptosomes prelabeled with [^{3}H]NA, [^{3}H]DA, [^{3}H]5-HT and [^{3}H]choline, respectively. When the pH of the superfusion medium was transiently lowered to 6.9 or 6.4, no significant changes in the tritium release were detected. Increasing the pH to 8.0 elicited a moderate, not significant, increase of tritium release. The releases of tritium were significantly augmented only when the pH of the superfusion medium was raised to 8.8, hardly compatible with any pathological condition. The releases, expressed as % of induced overflows, amounted to: 1.31 ± 0.12% ([^{3}H]NA); 0.74 ± 0.08% ([^{3}H]DA); 0.49 ± 0.11% ([^{3}H]5-HT); and 1.41 ± 0.19% ([^{3}H]ACh).

Effects of pH changes on the release of [^{3}H]neurotransmitters evoked by AMPA

It was previously shown that hippocampal noradrenergic and serotonergic, striatal dopaminergic and cortical cholinergic nerve endings are endowed with presynaptic receptors of the AMPA type, whose activation induces a Ca^{2+}-dependent, exocytotic-like release of NA, DA, 5-HT and ACh (Desce *et al*., 1991; Fink *et al*., 1995; Pittaluga *et al*., 1997; Ghersi *et al*., 2003). As reported in Figure 2, exposure of synaptosomes to 100 μM AMPA, at pH 7.4, evoked release of the four [^{3}H]neurotransmitters under study; the releases expressed as AMPA-induced tritium overflow, amounted to 0.55 ± 0.07 ([^{3}H]NA); 0.29 ± 0.04 ([^{3}H]DA); 0.33 ± 0.03 ([^{3}H]5-HT); and 0.37 ± 0.04 ([^{3}H]ACh), respectively, confirming results previously reported by the above authors.

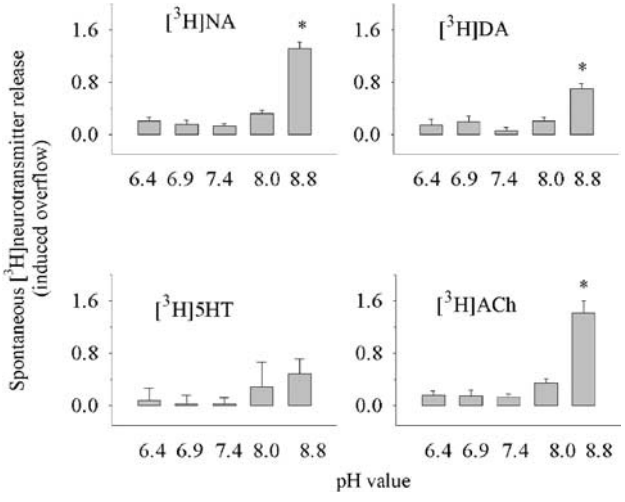

Figure 1 Effects of pH on the spontaneous release of neurotransmitters from superfused synaptosomes. Hippocampal synaptosomes were prelabeled with [^{3}H]NA or [^{3}H]5-HT, striatal synaptosomes with [^{3}H]DA and cortical synaptosomes with [^{3}H]choline. Synaptosomes were exposed to superfusion media of different pH, for 90 s. Superfusion was then continued with standard medium (pH 7.4) till the end of the experiment. Results are expressed as induced overflow. Data are mean$\pm$s.e.m. of four experiments run in triplicate (three superfusion chambers for each experimental condition). *$P<0.05$ *versus* control.

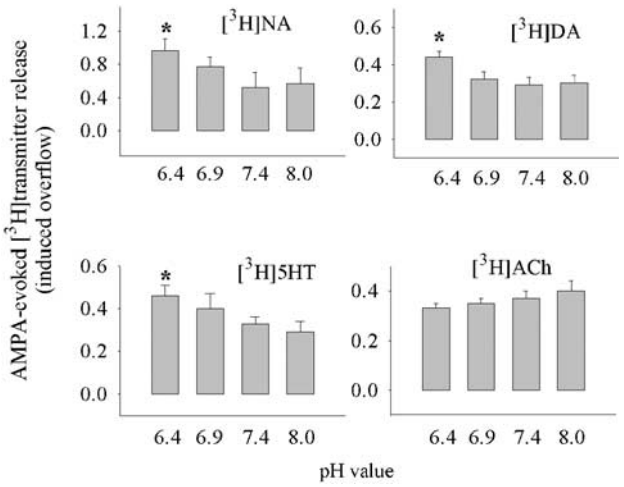

Figure 2 Effects of pH on the AMPA-evoked release of transmitters from superfused synaptosomes. Synaptosomes were exposed to 100 μM AMPA for 90 s concomitantly with media of different pH. Superfusion was then continued with standard medium (pH 7.4) till the end of the experiment. Results are expressed as induced overflow. Data are mean$\pm$s.e.m. of three to seven experiments run in triplicate. *$P<0.05$ *versus* control.

Figure 2 illustrates the pH-dependency of the AMPA-evoked releases. Transient changes (90 s) in the pH of the superfusion medium were applied concomitantly with AMPA. Lowering pH from 7.4 to 6.9 produced slight, not significant, increase of the AMPA-evoked release. However, 90 s application of medium at pH 6.4 significantly potentiated the AMPA-evoked overflow of [^{3}H]NA and [^{3}H]5-HT from hippocampal synaptosomes as well as that of [^{3}H]DA from striatal nerve endings. In contrast, the AMPA-evoked overflow of [^{3}H]ACh from cortical synaptosomes remained unaffected. When the [H^+] was decreased (pH 8.0), no significant changes in the AMPA-evoked release could be observed (Figure 2).

Calcium-dependency and vesicular origin of the AMPA-evoked release of [^{3}H]neurotransmitters at different pH

It had been reported that activation of AMPA presynaptic receptors at physiological pH (pH 7.4) elicits the release of the [^{3}H]transmitters under study in an external Ca^{2+}-dependent manner (Pittaluga *et al*., 1997). Results in Table 1 confirm these findings. Moreover the table also shows that the release of the neurotransmitters evoked by 100 μM AMPA at pH 6.4 is prevented by omission of external Ca^{2+}, suggesting that extracellular H^+ modulates the calcium-dependent component of the AMPA-evoked neurotransmitter release.

The vesicular origin of the releases induced by AMPA is shown by results in Table 2. Both at pH 7.4 and 6.4, the AMPA-evoked effects were almost totally abolished when the

Table 1 Ca^{2+}-dependency of the AMPA-evoked release of neurotransmitters at different pH

	pH = 7.4		*pH = 6.4*	
	1.2 mM Ca^{2+}	*No Ca^{2+}*	*1.2 mM Ca^{2+}*	*No Ca^{2+}*
[^{3}H]NA	0.65±0.04	−0.14±0.08*	0.92±0.03*	0.01±0.31*
[^{3}H]DA	0.28±0.04	0.04±0.09*	0.48±0.03*	−0.04±0.03*
[^{3}H]5-HT	0.53±0.08	0.05±0.08*	0.90±0.11*	0.03±0.03*
[^{3}H]Ach	0.58±0.16	0.05±0.17*	0.46±0.06	0.06±0.05*

Synaptosomes were superfused as described in Methods. When indicated, at $t = 20$, synaptosomes were exposed to a Ca^{2+}-free EGTA (0.5 mM)-containing medium. AMPA (100 μM) was added concomitantly with media at pH = 7.4 or 6.4 for 90 s; superfusion was then continued with standard medium (pH 7.4) till the end of the experiment. Results are expressed as evoked overflow (see Methods). Data are means±s.e.m. of three experiments run in triplicate. *$P<0.05$ *versus* respective control.

Table 2 Effects of bafilomycin A1 on the AMPA-evoked neurotransmitter release at different pH

	pH = 7.4		*pH = 6.4*	
	Control	*Bafilomycin A1*	*Control*	*Bafilomycin A1*
[^{3}H]NA	0.58±0.10	−0.07±0.08*	1.12±0.07*	0.19±0.10*
[^{3}H]DA	0.37±0.05	0.08±0.05*	0.52±0.05*	0.06±0.09*
[^{3}H]5-HT	0.57±0.09	0.13±0.07*	1.13±0.16*	−0.06±0.11*
[^{3}H]ACh	0.33±0.08	0.09±0.07*	0.28±0.04	0.05±0.03*

Synaptosomes were preincubated with bafilomycin A1 (0.1 μM) and subsequently labelled with the radiotracers. Synaptosomes were then exposed in superfusion to 100 μM AMPA concomitantly with media at pH = 7.4 or 6.4 for 90 s; superfusion was continued with standard medium (pH 7.4) till the end of the experiment. Results are expressed as evoked overflow. Data are means±s.e.m. of three to four experiments run in triplicate. *$P<0.05$ *versus* respective control.

vesicular storage of neurotransmitters was prevented by pretreating synaptosomes with bafilomycin A1, a selective blocker of the vesicular ATPase. Thus, AMPA receptor activation causes release of the vesicular pool of transmitters in an external Ca^{2+}-dependent manner, compatible with an exocytotic-like process.

Accordingly, the results reported in Table 3 show that outflow of cytoplasmic transmitters through plasmamembrane transporters working in the reverse mode is unlikely to occur, under our experimental conditions. In fact, independently of the extraterminal pH, the AMPA-evoked releases of [^{3}H]NA, [^{3}H]DA, [^{3}H]5-HT and [^{3}H]ACh were unaffected by the presence of the selective transporter blockers nisoxetine, GBR12909, 6-nitroquipazine and hemicholinium-3. These compounds did not modify, on their own, the spontaneous transmitter release (not shown).

Table 3 Effect of transporter inhibitors on the AMPA-evoked release of neurotransmitters at different pH

		pH 7.4	*pH 6.4*
[^{3}H]NA	Control	0.65±0.04	1.02±0.03*
	+0.1 μm nisoxetine	0.72±0.14	1.28±0.10*
[^{3}H]DA	Control	0.31±0.04	0.51±0.06*
	+0.1 μm GBR12909	0.32±0.06	0.49±0.12*
[^{3}H]5-HT	Control	0.64±0.08	0.96±0.09*
	+0.1 μm 6-nitroquipazine	0.60±0.10	0.86±0.06*
[^{3}H]ACh	Control	0.58±0.16	0.46±0.06
	+10 μm hemicholinium-3	0.50±0.07	0.57±0.05

Synaptosomes were exposed in superfusion for 90 s to AMPA (100 μM) alone or to AMPA plus the selective uptake inhibitors concomitantly with media at different pH. Superfusion was then continued with standard medium (pH 7.4) till the end of the experiment. Results are expressed as evoked overflow. Data are mean±s.e.m. of three experiments run in triplicate. *$P<0.05$ *versus* control.

Effects of pH changes on the K^+-evoked neurotransmitter release

The changes in the AMPA-evoked neurotransmitter release observed might have been due to facilitation by H^+ ions of some of the steps of the exocytotic machinery. We therefore examined the effects of protons on the release evoked by a depolarizing stimulus known to elicit transmitter exocytosis. Synaptosomes were exposed for 90 s to KCl (15 mM)-enriched media at different pH (6.4–8.0). Superfusion with high-K^+ medium at pH 7.4 provoked quantitatively different [^{3}H] transmitter overflows, which amounted to 11.81±1.01 ([^{3}H]NA); 1.13±0.06 ([^{3}H]DA); 9.48±0.86 ([^{3}H]5-HT) and 2.95±0.06 ([^{3}H]ACh), respectively (Figure 3). The figure also shows that, when the external pH was lowered to 6.8 or 6.4, the K^+-evoked overflows of the four [^{3}H]transmitters were decreased. Increasing the pH from 7.4 to 8.0 slightly, although not significantly, increased the K^+-evoked overflows.

When comparing results in Figure 2 and Figure 3, a large quantitative disparity between AMPA- and K^+-evoked overflows can be observed. Release evoked by 15 mM K^+ has often been shown to be Ca^{2+}-dependent and exocytotic. Since K^+-depolarization includes the whole nerve ending membrane, whereas that caused by AMPA is probably localized in the vicinity of the Na^+-permeant AMPA channel, it is conceivable that high-K^+ activates more voltage-sensitive Ca^{2+} channels than AMPA and consequently elicits higher release. Clearly, other explanations may exist for the difference between K^+- and AMPA-evoked release.

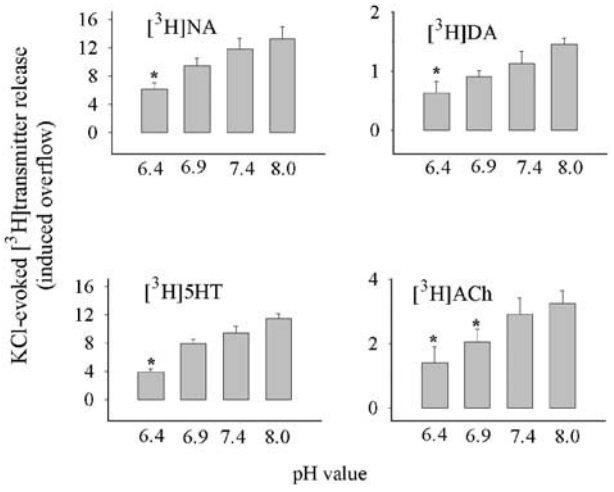

Figure 3 Effects of pH on the KCl-evoked release of transmitters from superfused synaptosomes. Synaptosomes were exposed for 90 s to K^+-enriched (15 mM) media of different pH. Superfusion was then continued till the end of the experiment with standard medium (pH 7.4). Results are expressed as induced overflow. Data are mean ± s.e.m. of three to four experiments run in triplicate. $*P<0.05$ *versus* control.

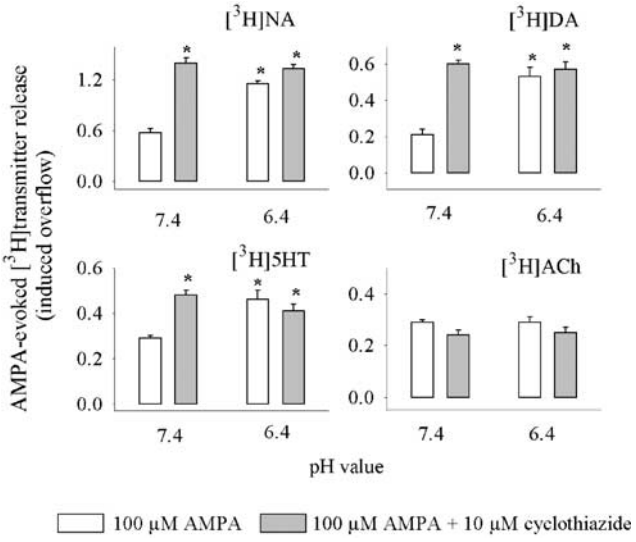

Figure 4 Effects of protons and cyclothiazide on the AMPA-evoked release of neurotransmitters from superfused synaptosomes. Synaptosomes were exposed for 90 s to AMPA alone or to AMPA plus cyclothiazide concomitantly with media of different pH. Superfusion was then continued with standard medium (pH 7.4) till the end of the experiment. Results are expressed as induced overflow. Data are mean ± s.e.m. of three to five experiments run in triplicate. $*P< 0.05$ *versus* control.

Effects of pH changes and cyclothiazide on the AMPA-evoked transmitter release

Figure 4 shows that, at pH 7.4, the AMPA-evoked release of [^{3}H]NA, [^{3}H]DA and [^{3}H]5-HT was significantly enhanced when cyclothiazide was added to the superfusion medium concomitantly with AMPA. The figure also shows that, while acidification to pH 6.4 potentiated the AMPA-evoked release of [^{3}H]NA, [^{3}H]DA and [^{3}H]5-HT, at this acidic pH cyclothiazide lost its ability to potentiate the AMPA-evoked overflow of the three [^{3}H] neurotransmitters. In contrast, the AMPA-induced release of [^{3}H]ACh was found to be insensitive to cyclothiazide both when superfusing synaptosomes with medium at pH 7.4 or when extraterminal pH was lowered to 6.4 (Figure 4).

Discussion

Previous works aimed at the identification and characterization of native AMPA receptors reported the existence, in different areas of the rat brain, of presynaptic AMPA/kainate receptors located on nerve terminals where they mediate enhancement of the Ca^{2+}-dependent exocytotic-like release of various neurotransmitters (Desce *et al*., 1991; Pittaluga & Raiteri, 1992; Fink *et al*., 1995; Pittaluga *et al*., 1997).

Receptors present on different neurons were found to desensitize differently (Pittaluga *et al*., 1997). In particular, the AMPA-evoked releases of NA, DA and 5-HT all were potentiated by cyclothiazide, a drug able to prevent desensitization of AMPA receptors (Partin *et al*., 1993; Barnes *et al*., 1994; Bettler & Mulle, 1995), but not by concanavalin A, which preferentially inhibits desensitization of kainate receptors (Partin *et al*., 1993; Bettler & Mulle, 1995). The AMPA- or kainate-evoked release of glutamate was sensitive to concanavalin A only. The AMPA-evoked release of ACh and GABA was insensitive to either cyclothiazide or concanavalin A (Pittaluga *et al*., 1997).

More recently, the pharmacology of the receptors mediating the release of NA, DA, 5-HT and ACh was investigated by using a number of selective AMPA and kainate receptor antagonists (Ghersi *et al*., 2003). The differential patterns of the antagonists tested led to conclude that (i) the four receptors involved, including the cyclothiazide-insensitive receptor mediating release of ACh, belong to the AMPA type; and (ii) the AMPA receptors studied represent four pharmacologically distinct receptors possibly due to their different subunit composition.

The major finding of the present investigation is that reduction of external pH to levels of acidity characteristic of ischemia *in vivo* (Siesjö, 1988; Tombaugh & Sapolsky, 1993) potentiated the AMPA-evoked release of various neurotransmitters. The AMPA-evoked releases of NA, DA and 5-HT, but not that of ACh, were enhanced when the extraterminal pH was decreased from 7.4 to 6.4. As the spontaneous releases of the transmitters tested were not affected by pH changes, acidification appears to exclusively affect the overflows of NA, DA and 5-HT provoked by AMPA.

Increases of extraterminal [H^+] could lead to augmentation of the AMPA-evoked transmitter release through various mechanisms. Acidification could facilitate an AMPA receptor-mediated efflux of transmitters by Ca^{2+}-independent plasmamembrane transporter reversal (Attwell *et al*., 1993; Levi & Raiteri, 1993). Accordingly, acidification enhanced the AMPA-evoked release of NA, DA and 5-HT, but not that of ACh, for which no plasmamembrane transporters exist. However, the effects of pH were strictly Ca^{2+}-dependent and insensitive to transporter blockers, indicating that a carrier-mediated release process is not involved. On the other hand, the Ca^{2+}-dependent effects of protons on the AMPA-evoked release of NA, DA and 5-HT disappeared when the vesicular

storage of the transmitters had been prevented by bafilomycin A1, consistent with an exocytotic mechanism. Acidification could therefore positively affect some steps of the exocytotic release elicited by AMPA depolarization. However, such a mechanism seems unlikely, because the exocytotic releases of NA, DA and 5-HT, as well as that of ACh, evoked by depolarization with relatively low [K^+] (15 mM) was not enhanced, actually it was inhibited, when extraterminal pH was lowered to 6.4. The findings that acidification reduced the exocytosis evoked by high-K^+ of all the four transmitters tested, whereas the proton-induced potentiation differentially affected the release of the four transmitters (the AMPA-evoked ACh release was H^+-resistant) suggest that protons act at different targets when release is stimulated by high-K^+ or by AMPA.

These considerations together with the reported different desensitization properties of the four presynaptic AMPA receptors under study (Pittaluga *et al*., 1997) suggest that the function of the cyclothiazide-sensitive AMPA receptors mediating release of NA, DA and 5-HT, but not that of the cyclothiazide-insensitive receptor mediating the release of ACh, is enhanced by acidification. Controversial results are present in the literature concerning effects of H^+ on AMPA receptors. Evidence has been provided supporting a proton-mediated inhibition of AMPA/kainate receptor function (Christensen & Hida, 1990; Traynelis & Cull-Candy, 1991; Traynelis *et al*., 1995; Ihle & Patneau, 2000; Lei *et al*., 2001). On the other hand, H^+ ions were shown to augment excitotoxicity produced by AMPA/kainate receptor activation (McDonald *et al*., 1998; Mott *et al*., 2003).

Glutamate receptors of the NMDA type mediating transmitter release exist on rat hippocampal (Pittaluga & Raiteri, 1990) and cortical (Fink *et al*., 1990) noradrenergic axon terminals, on striatal dopaminergic nerve endings (Roberts & Anderson, 1979; Krebs *et al*., 1991) and on cortical serotonergic nerve endings (Fink *et al*., 1995). Release-enhancing AMPA and NMDA receptors were shown to coexist on the same noradrenergic (Pittaluga & Raiteri, 1992) or dopaminergic (Krebs *et al*., 1991) nerve terminal, where activation of AMPA receptors permits activation of NMDA receptors in the presence of physiological concentrations of Mg^{2+}.

Protons are known to inhibit NMDA receptor function (Traynelis & Cull-Candy, 1990). Variants of the NR1 subunit contain a pH sensor. The H^+ inhibition is strongly reduced when the exon-5 insert N1 is present in the NR1 subunit (Traynelis *et al*., 1995). We found that the NMDA receptor sited on hippocampal noradrenergic nerve terminals is H^+-sensitive, its function being abrogated at pH 6.6. However, the NMDA receptors present on dopaminergic terminals is H^+-insensitive (Pittaluga *et al*., 2001), probably due to the presence of the N1 cassette in the NR1 subunit. As to the sensitivity of AMPA receptors to H^+, Ihle & Patneau (2000) found in cultured cells that receptors containing the *flop* isoform exhibited more extensive desensitization and were more potently inhibited by protons, whereas receptors containing *flip* isoforms were more resistant to desensitization and poorly sensitive to extracellular acidification. It is at present unknown if the native release-regulating AMPA receptors here characterized are differentially sensitive to protons and cyclothiazide and desensitize differently upon exposure to agonists (Pittaluga *et al*., 1997 and Figure 4) because of the presence of *flop* or *flip* isoforms.

Whatever the mechanisms, it seems that the AMPA and NMDA receptors coexisting on hippocampal noradrenergic and striatal dopaminergic terminals are differentially sensitive to pH changes: protons potentiate the function of AMPA receptors, but depress or leave unmodified that of NMDA receptors. If this also occurs at the postsynaptic level where AMPA and NMDA receptors coexist, it would bear pathological implications. It is known that extracellular pH can decline to $\sim$6.5 during ischemia or hypoxia (Siemkowicz & Hansen, 1981; Siesjö, 1988; Chesler & Kaila, 1992). The reduction in the NMDA receptor function at this acidic pH has been proposed to represent a kind of protection against excitotoxicity (Giffard *et al*., 1990; Tombaugh & Sapolsky, 1990; Kaku *et al*., 1993). The present data showing that, at pH 6.4, a number of AMPA receptor subtypes become more responsive than at physiological pH would imply that acidity may not necessarily be neuroprotective for the neurons on which AMPA receptors exist. Interestingly, pharmacological blockade of AMPA receptors, but not of NMDA receptors, was reported to produce substantial protective effects in the ischemic brain (Buchan *et al*., 1991; Le-Peillet *et al*., 1992; Pellegrini-Giampietro *et al*., 1992; Sheardown *et al*., 1993).

The anti-desensitization drug cyclothiazide and extraterminal acidification potentiated almost to the same extent the AMPA-evoked release of NA, DA and 5-HT, while leaving unaffected the AMPA-evoked release of ACh. Moreover, the effects of AMPA on the release of NA, DA and 5-HT, once potentiated by increasing extraterminal [H^+], could not be further enhanced by coapplication of cyclothiazide. Although the present results do not permit to conclude that protons bind on desensitizing AMPA receptors where cyclothiazide binds, they suggest that protons interact with the AMPA receptor desensitization mechanism and might represent endogenous agents able to prevent desensitization of some, but not all, AMPA receptors.

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