Regional and Subunit-Specific Downregulation of Acid-Sensing Ion Channels in the Pilocarpine Model of Epilepsy

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Acid-sensing ion channels (ASICs) constitute a recently discovered family of excitatory cation channels, structurally related to the superfamily of degenerin/epithelial sodium channels. ASIC1b and ASIC3 are highly expressed in primary sensory neurons and are thought to play a role in pain transmission related to acidosis. ASIC1a, ASIC2a, and ASIC2b are also distributed in the central nervous system where their function remains unclear. We investigated here the regulation of their expression during status epilepticus (SE), a condition in which neuronal overexcitation leads to acidosis. In animals treated with pilocarpine (380 mg/kg) to induce SE, we observed a marked decrease of ASIC2b mRNA levels in all hippocampal areas and of ASIC1a mRNA levels in the CA1-2 fields. These changes were also observed after protective treatment from neuronal cell death with diazepam (10 mg/kg) and pentobarbital (30 mg/kg). These findings suggest a key role of channels containing ASIC1a and ASIC2b subunits in both normal and pathological activity of hippocampus.

INTRODUCTION

Mammalian proton-gated channels, named acid-sensing ion channels (ASICs), have been recently discovered and characterized in heterologous expression systems (see review in Waldmann & Ladunski, 1998). They belong to the superfamily of degenerins/epithelial sodium channels (DEG/ENaC) that are characterized by a topology with two membrane-spanning domains, a large cysteine-rich extracellular loop, and intracellular amino and carboxy termini (reviewed by Garcia-Anoveros & Corey, 1997). To date, five members of this family have been cloned in mammals: ASIC1a (also called brain sodium channel 2, BNaC2) (Garcia-Anoveros et al., 1997; Waldmann et al., 1997a), ASIC1b (Chen et al., 1998); ASIC2a (also called mammalian degenerin 1, MDEG1, or brain sodium channel 1, BNaC1) (Price et al., 1996; Waldmann et al., 1996); ASIC2b (also called MDEG2) (Lingueglia et al., 1997); and ASIC3 (also called dorsal root ganglia ASIC, DRASIC) (Waldmann et al., 1997b; De Weille et al., 1998; Babinski et al., 1999). These channels have the common feature of generating excitatory currents in response to decreasing pH when studied in vitro. As an exception, ASIC2b does not appear to respond to low pH applications when expressed in homomeric form; it is, however, capable of modulating the response of other ASIC subunits. Indeed, ASIC2b lowers the threshold of activation by protons of ASIC2a while it changes the ion selectivity of ASIC3 (Lingueglia et al., 1997). Although the composition
of native ASIC channels is still unknown, evidence for heteromultimeric channel formation with distinctive functional properties has been obtained (Bassilana et al., 1997; Babinski et al., 2000).

ASICs are highly expressed in peripheral and central nervous tissue with the exception of rat ASIC1b and ASIC3, which are almost exclusively localized or enriched in peripheral sensory neurons (Waldmann et al., 1997b; Chen et al., 1998); the other ASIC subunits have a widespread distribution in the adult rat brain (Waldmann et al., 1997a; Lingueglia et al., 1997). It has been reported that under normal conditions they are specifically expressed in neuronal cells (Waldmann et al., 1996; Lingueglia et al., 1997). High levels of mRNA have been localized in olfactory bulbs, cerebral neocortex, hippocampus, habenula, amygdala, and cerebellum for ASIC1a as well as for both ASIC2 variant subunits. Although in rodents ASICs have been detected exclusively in the nervous tissue, a more diffused localization in other tissues has been reported for human ASIC2a and ASIC3 (Babinski et al., 1999, 2000). Generally, these channel subunits appear to be highly conserved between species, suggesting an involvement in important regulatory functions (Waldmann & Lazdunski, 1998).

A role in chemical pain sensation, especially that associated with increased local acidification, has been proposed for ASIC subunits localized in sensory neurons (Waldmann & Lazdunski, 1998; McCleskey & Gold, 1999). In the central nervous system, their function is still hypothetical. However, proton-activated neuronal currents have been identified in different brain areas (reviewed in Chesler & Kaila, 1992; Akaike & Ueno, 1994; Deitmer & Rose, 1996). Thus, ASIC receptors may function as low pH detectors in normal as well as pathophysiological conditions such as cerebral ischemia or epilepsy, where tissue acidification occurs (Kraig et al., 1983; Siensjo et al., 1985; De Curtis et al., 1998; Xiong et al., 2000). Interestingly, the sensitivity and channel response to pH of ASIC1a, ASIC1b, and ASIC3 in sensory neurons can be modulated by the neuropeptide FF and FMRFamide (Askwith et al., 2000). Hence central ASIC receptors may similarly be regulated. Gain-of-function mutations of ASIC2a and ASIC2b (MDEG channels) potentially causing cell death have also been detected and have been proposed to participate in neurodegenerative diseases (Lingueglia et al., 1997; Adams et al., 1998; Champigny et al., 1998). Despite the potential interest of understanding the regulation of ASIC receptors in pathophysiological conditions, until now no experiments have been designed to investigate their role in brain dysfunction. To this aim, we studied the mRNA expression pattern of central ASIC1a, ASIC2a, and ASIC2b subunits in a well-established model of epilepsy, based on treatment with the cholinergic agent pilocarpine (Turski et al., 1983; Clifford et al., 1987; Liu et al., 1994).

MATERIALS AND METHODS

Animals and Treatments

Sprague–Dawley male rats (225–250 g body weight) were used. They were housed, two per cage, under controlled temperature (23 ± 1°C), humidity (40–60%), and daylight cycle (light from 7:00 AM to 7:00 PM). In a first series of experiments, animals were divided in two groups of treatment, the first (n = 12) receiving an intraperitoneal (ip) injection of pilocarpine (380 mg/kg) to induce seizures and status epilepticus (SE), and the second (n = 6) treated with saline, representing the control group. To prevent discomfort caused by the activation of peripheral muscarinic receptors by pilocarpine, all the animals were pretreated with scopolamine methylbromide (1 mg/kg ip 30 min before the pilocarpine injection). The animals from this experiment were used to study ASIC mRNA levels by Northern blot hybridization 15 min, 2 h, and 24 h (n = 6 for each time interval) after pilocarpine or saline injection. A second series of experiments was designed to analyze the changes in regional distribution of ASIC mRNA levels in the brain 24 h after pilocarpine treatment. In these experiments (n = 4–10 per group), three additional treatment groups were considered: a group treated with pilocarpine at a subconvulsive dose (200 mg/kg ip), another receiving the convulsive dose of pilocarpine followed by a combination of diazepam (10 mg/kg ip) and pentobarbital (30 mg/kg ip) to stop seizures after 30 min of SE, and finally one group treated only with the diazepam and pentobarbital combination. This last treatment has been shown to prevent efficaciously neuronal damage in the pilocarpine model (Lemos & Cavaleiro, 1995) and thus has been adopted to discriminate the decrease of mRNA levels due to damage caused by downregulation.

The animals receiving a convulsive dose of pilocarpine were monitored for at least 2 h for the scoring of convulsions and for determining the effects of anticonvulsive treatments. The classification of Golaraei et al. (1992) into five categories of seizure activity was adopted. In particular, hypoactivity corresponded to
stage I, monoclonal jerks of the head, head bobbing, and facial automatism to stage II, whole body bilateral activity resembling wet dog shakes to stage III, rearing of forelimbs to stage IV, and generalized clonic–tonic activity and loss of posture to stage V. SE was defined as an uninterrupted stage V activity for at least 30 min. At the end of the scheduled time intervals from pilocarpine injection, the animals were sacrificed by decapitation and brains were rapidly extracted. For Northern blot analysis, cerebral cortices and hippocampi were rapidly dissected out and immediately frozen on dry ice powder at −80°C. For in situ hybridization and histology, brains were frozen briefly in liquid isopentane at −35°C and then stored at −80°C.

Synthesis and Labeling of Probes

Full-length cDNAs for rat ASIC1a (ASIC; GenBank Accession No. U94403), ASIC2a (MDEG1; GenBank Accession No. U53211), and ASIC3 (DRASIC; GenBank Accession No. AF013598) were amplified by RT-PCR using the Expand enzyme mix (Roche Diagnostics) using forward and reverse primers that included the initial methionine and stop codons, respectively. Amplified cDNAs were then directionally subcloned into the pcDNA3 eukaryotic expression vector (Invitrogen) and used to prepare the specific riboprobes for hybridization studies. Each probe was designed from a DNA coding region presenting sufficiently low homology with respect to the other ASIC subunits, as can be appreciated by the comparison of ASIC sequences shown in Fig. 1 and from the subcloning strategy illustrated in Fig. 2. For ASIC1a, a 344-bp fragment corresponding to nucleotides 538–881 of the coding region (Ser538–Met881) was subcloned between PstI and NcoI sites of the pGEM5zf vector (Promega). For ASIC2a, a 279-bp fragment corresponding to nucleotides 181–459 (Ser181–Met459) was subcloned between SacI and SphI sites of the pGEM5zf vector. A 379-bp fragment of ASIC3, corresponding to nucleotides 1142–1520 (Leu1142–Pro1520), was subcloned between SacI and ApaI sites of the pGEM5zf vector. The probe for ASIC2b (MDEG2; GenBank Accession No. Y14635) was isolated from a partial mouse ASIC2b EST clone (Research Genetics, GenBank Accession No. W50528). A 417-bp fragment equivalent to nucleotides 267–683 of the rat sequence (97% identity: Val267–Leu683) was subcloned into the pGEM5zf vector between Sall and SacI. Sense and antisense cRNAs were synthesized respectively with the SP6 and T7 RNA polymerases for ASIC1a, ASIC2a, and ASIC3 and the T7 and SP6 polymerases for ASIC2b (see Fig. 2) in the presence of [32P]UTP for Northern blot or a mix of 35S-CTP and 35S-UTP for in situ hybridization.

RNA Preparation and Northern Blot Analysis

As previously described (Meyer et al., 1996), animals belonging to a common treatment group were pooled (n = 2 for saline treatment per each time interval; n = 3–4 for pilocarpine treatment per each time interval) and total RNA was extracted from cerebral cortices (two samples/animal) and hippocampi (two samples/animal) with the TRIzol reagent (Life Technologies, Gaithersburg, MD). Approximately 10 μg of total RNA was separated by electrophoresis on a 1.2% agarose gel containing 20 mM Hepes (pH 7.8), 1 mM EDTA, and 6% formaldehyde and then transferred to a nylon membrane of 0.45-μm pore (Nytran Plus, Schleicher & Schuell, Keene, NH) by capillary action. After blotting, membranes were dried and RNA was fixed by UV irradiation. The 18S and 28S ribosomal RNA species and each marker in the RNA ladder ( Gibco BRL, Burlington, Ontario, Canada) were visualized by staining membranes with 0.02% methylene blue in 0.3 M sodium acetate (pH 5.5). The filters were prehybridized at 65°C for 90 min in a solution composed of 5% sodium dodecyl sulfate (SDS), 0.4 M Na2PO4, 1 mM EDTA, 0.1% bovine serum albumin (BSA), and 50% formamide and then hybridized overnight at 65°C in the presence of a [32P]UTP cRNA probe (see Fig. 2). After hybridization, filters were washed in a sodium citrate-based buffer: 0.1× SSC (1.5 M NaCl, 0.15 M sodium citrate), 0.1% SDS, and 1 mM EDTA at 70°C for 2 h, wrapped in plastic, and exposed at −70°C for different time intervals to X-ray film (Kodak, Rochester, NY) with an intensifying screen. The same filters were also rehybridized with a rat 18S riboprobe to standardize for the amount of mRNA in each lane. Appropriate exposure times of all autoradiograms were quantified using a Shimadzu CS-9000 densitometer (exposure times were 5 h, 15 h, 24 h, 3 days, and 5 days; a 24-h exposure was found to be appropriate for every probe used).

In Situ Hybridization and Image Analysis

The procedure of in situ hybridization has previously been described in detail (Marcinkiewicz et al., 1997). Three saline-treated controls, four 380 mg/kg pilocarpine-treated and five pilocarpine and
diazepam-pentobarbital-treated rats, and three diazepam-pentobarbital-treated and four 200 mg/kg pilocarpine-treated rats were used. Briefly, 6-μm-thick sections were fixed for 1 h with 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2) and washed extensively with phosphate-buffered saline (PBS) and then reacted with acetic anhydride in 0.1 M triethanolamine solution. Then, the sections were hybridized overnight at 55°C using double $^{35}$S-CTP- and $^{35}$S-UTP-labeled cRNA probes corresponding to the cDNAs shown in Fig. 1. After extensive washing, the sections were dried and exposed to X-ray film for 2–3 days.

Autoradiographic films were then analyzed by microdensitometry as previously described (Fuxe et al., 1996; Biagini et al., 1998). Four sections for each animal were analyzed and averaged for statistical analysis. Briefly, the value of nonspecific mean gray tone was measured in the area of the film immediately outside the slice, close to the area of cerebral cortex eventually analyzed. An area of corpus callosum was taken as index of background labeling. Transmittance percentage values (T%) of total and nonspecific staining were then obtained by dividing the mean gray tone value of every area analyzed by the mean gray tone value of the background. Optical density (OD) values were then calculated according to the formula OD = $-\log T\%$, for both nonspecific gray tone values and specifically labeled areas. The specific OD was obtained by deducting nonspecific OD from total OD for each studied region. To ensure that microdensitometric analysis was carried out in the linear range of X-ray film sensitivity, the film response curve was previously established by using $^3$H-labeled microscales of known radioactivity (Amersham) and plotting OD vs DPM/area values. The appropriate exposure time for each ASIC probe (2 days for ASIC1a, 3 days for ASIC2a and ASIC2b) was then chosen in order to keep the signal within the linear range (around 50% of saturation values) of X-ray film and adjusting specific activity for the various probes.

Histological Procedures and Neuropathological Evaluation

The lesion provoked by SE was evaluated in the hippocampal region 24 h after pilocarpine injections. Hematoxylin–eosin staining was performed on 6-μm-thick cryostat sections fixed for 60 min in 4% phosphate-buffered formaldehyde and washed overnight in water. According to Fujikawa (1996), acidophilic neurons were considered as irreversibly damaged cells and counted as such in each hippocampal field by a collaborator unaware of the experimental design.

Statistical Analysis

Data from microdensitometric quantification of in situ hybridization autoradiograms were analyzed by one-way analysis of variance followed by Dunnett’s test for multiple comparisons. Data from the neuropathological evaluation of acidophilic cells were analyzed by Student’s t test. Results are presented as means ± SD; $P < 0.05$ was chosen as the threshold for statistical significance.

RESULTS

Distribution of ASIC mRNA in the Brain

As shown in Figs. 1 and 2, different mRNA transcripts were specifically identified by Northern blot hybridization with the various ASIC riboprobes. In particular, the ASIC1a probe hybridized two transcripts of approximately 4.3 and 3.2 kb in length. Similar multiple transcripts with ASIC1a-specific riboprobes have also been reported by other authors (Chen et al., 1998). The transcripts identified with ASIC2a, ASIC2b, and ASIC3 probes corresponded to those already described in the literature (Waldmann et al., 1996, 1997b; Lingueglia et al., 1997).

The distribution of ASIC1a, ASIC2a, and ASIC2b in the central nervous system has been described as widespread and largely overlapping (Lingueglia et al., 1997). Accordingly, we observed that they are codistributed in olfactory bulbs, cerebral neocortex, piriform cortex, hippocampal formation, amygdala, and cerebellum (Figs. 3 and 4). In addition, we found that all three subunits were coexpressed in olfactory tuber
cle, habenula, hypothalamus, dorsal raphe, and central gray. However, in each of these regions every subunit presented different levels of expression and sometimes a specific anatomical localization. This observation was particularly striking in the hippocampus, where ASIC1a was uniformly expressed (Figs. 3B, 4B, and 4C), ASIC2a was barely detectable (Figs. 3D, 4E, and 4F), while ASIC2b was expressed at higher levels in the CA3 hippocampal field (Figs. 3F, 4H, and 4I). At higher resolution (Fig. 5), ASIC1a appeared to be the prevalent subunit in pyramidal cells of the CA1 field, in which ASIC2b was also expressed. In contrast, this relation was reversed in CA3 pyramidal neurons.
and mossy cells of the dentate hilus, where ASIC2b prevailed over ASIC1a. In basket cells and granule cells of the dentate gyrus, ASIC1a was again expressed at a higher level than ASIC2b. No signal for ASIC2a mRNA was detected in silver grain-labeled hippocampal sections.

In other brain regions, all three ASIC subunits were not always coexpressed, but their expression appeared to be limited to only one or two subunits. For instance, ASIC1a was expressed at a relatively high level in the caudatus–putamen where ASIC2a was faint and ASIC2b mRNA levels were barely detectable (Fig. 4B vs Figs. 4E and 4H). Similar results were also seen in the superior and inferior colliculi (Fig. 4A vs Figs. 4D and 4G). Interestingly, ASIC2a was expressed at very high levels in the medial habenular nucleus and in septofimbrial and triangular nucleus (also grouped as posterior septal nuclei; Fig. 4D), the latter constituting a rich source of purinergic innervation for the habenular region (Sperlagh et al., 1998). High levels of ASIC2a mRNA were also seen in the zona incerta (Figs. 3C, 3D, 4E, and 4F). In the hypothalamus, ASIC2a was expressed at higher levels in the arcuate nucleus and in the “core” region of the ventromedial hypothalamic nucleus (Fig. 4F) and at lower levels in the remaining hypothalamic structures. Interestingly, ASIC1a and ASIC2b were both present in the “shell” region of the ventromedial hypothalamic nucleus (Figs. 4C and 4I), while in the arcuate nucleus all three subunits colocalized. Low to medium levels of expression for ASIC2a could also be appreciated in the pineal gland (Fig. 4D) and thalamic reticular nucleus (Figs. 3D and 4F). In the latter structure, ASIC1a also appeared to be present (Figs. 3B and 4C).

FIG. 1. Multiple alignment of the amino acid sequences of rat ASIC subunits. Residues conserved in all four rat sequences are boxed. The regions printed white on black correspond to the sequences of subunit-specific riboprobes.
ASIC2b distribution overlapped to a large extent with that of ASIC1a (see Fig. 3F vs Fig. 3B, and Fig. 4H vs Fig. 4B). Particularly striking was the similar pattern of distribution in the olfactory nuclei, piriform cortex, amygdaloid region, and dorsal raphe (Fig. 3E vs Fig. 3A). However, ASIC2b distribution was characterized by higher levels of expression in the endopiriform nucleus (Fig. 4I) as well as in the hypothalamic region, especially in the paraventricular hypothalamic nucleus, the periventricular hypothalamic nucleus, the anterodorsal preoptic nucleus, and the preoptic area (Fig. 3E). Bed nuclei of the stria terminalis were also strongly labeled, as was the parafascicular thalamic nucleus (Fig. 3F). In the thalamus, the ventrobasal thalamic complex also presented a low level of expression for ASIC2b, but not for the other subunits (Fig. 4I). Interestingly, the analysis of the hippocampal region also revealed an effect of pilocarpine-induced SE on ASIC1a mRNA levels (Figs. 7A and 7B), although the decrease in mRNA expression was less drastic than that of ASIC2b (Figs. 7C and 7D).

Changes in mRNA Expression Following SE

Alterations in ASIC mRNA levels were investigated by Northern blot and in situ hybridization. No changes were found with Northern blot analysis of ASIC1a and ASIC2a mRNA levels following treatment with pilocarpine and seizures. In contrast, ASIC2b mRNA levels appeared to decrease already at 15 min after pilocarpine injection, especially in the hippocampus, persisting at low levels for at least 24 h after treatment (Fig. 6). These changes in mRNA content were further corroborated by data from in situ hybridization (Fig. 7). Interestingly, the analysis of the hippocampal region also revealed an effect of pilocarpine-induced SE on ASIC1a mRNA levels (Figs. 7A and 7B), although the decrease in mRNA expression was less drastic than that of ASIC2b (Figs. 7C and 7D).

FIG. 2. Subcloning strategy of the ASIC riboprobes used in the present study. A PGEM5zf vector has been used to subclone specific fragments for each ASIC subunit, as described in detail under Materials and Methods. The probes were then tested with Northern blot hybridization with total RNA extracted from rat cerebral cortex (ASIC1a, ASIC2a, ASIC2b) or trigeminal ganglia (ASIC3). ASIC, acid-sensing ion channel; BNaC, brain sodium channel; DRASIC, dorsal root ganglia ASIC; MDEG, mammalian degenerin; 18S, 28S, ribosomal RNA.
As determined by semiquantitative microdensitometric analysis of *in situ* hybridization autoradiograms, the expression of the different ASICs was affected by SE in a regional and subunit-specific manner (Fig. 8). ASIC1a mRNA optical densities decreased significantly (0.036 ± 0.007 vs 0.082 ± 0.013, \( P < 0.01 \)) in the CA1-2 hippocampal fields. Lower levels of mRNA hybridization were also observed in the CA3 area, but were not statistically significant. No significant changes were observed in cerebral neocortex, dentate hilus, dentate gyrus, and habenula. ASIC2a mRNA optical densities were determined only in the cerebral neocortex and habenula, since the hippocampal expression was deemed too low for a reliable analysis. No changes were found for this ASIC subunit. The most remarkable changes were found for ASIC2b mRNA levels. In fact, a decrease of ASIC2b expression was generalized to all hippocampal fields. This finding was particularly evident in the CA3 hippocampal field, dentate hilus, and dentate gyrus, where ASIC2b mRNA optical densities decreased significantly to approximately 50% of the basal values (0.024 ± 0.006 vs 0.039 ± 0.009, \( P < 0.05 \) for CA1-2; 0.033 ± 0.005 vs 0.064 ± 0.014, \( P < 0.05 \) for CA3; 0.029 ± 0.001 vs 0.063 ± 0.011, \( P < 0.01 \) for dentate hilus; 0.017 ± 0.002 vs 0.037 ± 0.012, \( P < 0.05 \) for dentate gyrus). In the cerebral cortex, a decreasing trend was also found, but did not reach statistical significance. No changes were found in the habenular region.

The comparison of the other treatment groups revealed that pilocarpine at subconvulsive doses as well as the combined diazepam + pentobarbital treatment had no effects on the mRNA levels of the ASIC subunits considered in this study. Furthermore, although the combined administration of diazepam and pentobarbital effectively interrupted SE, the pilocarpine-induced decreases in mRNA expression remained similar whether or not pilocarpine was followed by the neuroprotective treatment (Fig. 8).
Neuronal Damage Following SE

To determine the extent of damage caused by pilocarpine-induced convulsions, the various hippocampal regions were examined after hematoxylin–eosin staining. Acidophilic cells were counted in CA1-2 and CA3 hippocampal fields, dentate hilus, and dentate gyrus (Fujikawa, 1996). No acidophilic cells were observed in control animals or in diazepam- or pentobarbital- or 200 mg/kg pilocarpine-treated rats.

In animals subjected to SE, cell loss was clearly detectable in the various hippocampal fields, especially in the CA3 region (Fig. 9). The least damaged region among those studied was the dentate gyrus. The CA1-2 hippocampal fields were less consistently damaged than CA3. Acidophilic neurons were also found in animals having received the neuroprotective treatment, but the overall number of acidophilic cells was threefold higher in rats treated only with pilocarpine (134.8 ± 31.6 vs 43.3 ± 22.2, P < 0.05).

DISCUSSION

ASICs have been proposed as fast proton receptors in the peripheral as well as in the central nervous system (Waldmann et al., 1997b; Lingueglia et al., 1997;
Waldmann & Lazdunski, 1998). In the brain, ASICs could detect pH drops caused by release of neurotransmitters and may participate in the regulation of synaptic activity. Indeed, their widespread distribution suggests that they may be involved in the modulation of several neuronal systems and neurotransmitter pathways. ASIC1α is almost ubiquitously expressed and can potentially participate in the assembly of homomultimeric or heteromultimeric proton-gated channels in every brain region considered. In contrast, the ASIC2a subunit displays a region-specific distribution, with high levels of mRNA in the medial habenular nucleus, the triangular septal nucleus, the zona incerta, and the hypothalamic nuclei. This specific anatomical pattern suggests a possible involvement of ASIC2a-containing channels in the control of important visceral, endocrine, and behavioral functions, such as those regulated by the septal–habenular complex (Sperlagh et al., 1998). ASIC2b also displays a distinctive distribution in forebrain structures, such as the CA3 field, the medial amygdaloid complex, the dorsomedial hypothalamic nucleus, and the parafascicular nucleus and the ventrobasal complex of the thalamus.

These subunit-specific distribution patterns suggest that the native composition of ASIC receptor channels is regulated on a regional basis. Indeed, in areas where several ASIC subunits are colocalized, namely, in ol-

FIG. 6. Northern blot hybridization for ASIC2b in the cerebral cortex and hippocampus of animals treated with saline (Sal) or pilocarpine (380 mg/kg) to induce status epilepticus. Hybridization with the 18S ribosomal RNA is also shown in order to compare sample loading.

FIG. 7. In situ hybridization of ASIC1α (A, B) and ASIC2b (C, D) mRNA in coronal slices of rat brains from saline-treated (A, C) or 380 mg/kg pilocarpine-treated (B, D) animals. Calibration bar, 2 mm.
factory bulbs, piriform cortex, cerebral neocortex, entorhinal cortex, hippocampus, amygdala, hypothalamus, dorsal raphe, and cerebellum, they might assemble into heteromultimeric channels, with specific kinetics, ion selectivity, or sensitivity to protons (Bassilana et al., 1997; Lingueglia et al., 1997; Babinski et al., 2000). Especially in the hippocampus, the possibility of a coassembly of ASIC1a and ASIC2b subunits in native ASIC receptors is strongly supported by our findings regarding their pattern of cellular distribution. In other structures, ASICs might preferentially constitute homomultimeric channels or form channel complexes with other still undiscovered ASIC subunits. This could be the case for the ASIC1a subunit in the caudatus–putamen and collicular regions or for ASIC2a in the septal–habenular complex. Similarly, in the hypothalamic nuclei and other specific areas, such as the bed nuclei of the stria terminalis or the thalamic parafascicular nucleus, the unique high level of expression of ASIC2b, known as a modulatory subunit only, raises the possibility that ASIC2b assembles with yet unidentified ASIC or non-ASIC subunits or that channels containing ASIC2b subunits are activated by stimuli other than protons. However, in the CA3 field and the medial amygdaloid region, ASIC2b might play a modulatory role in ASIC receptor function by forming heteromeric channels with the other known ASIC subunits, as suggested by the expression levels of ASIC1a and ASIC2a subunits.

The pilocarpine model of SE potentially points to a critical role of the ASIC2b subunit in the regulation of ASIC receptors and thus in neuronal excitability in the hippocampal formation. Indeed, our results indicate that SE is associated with a consistent decrease of ASIC2b subunit mRNA levels in all hippocampal fields and to some degree also in the cerebral cortex. ASIC1a mRNA levels also decrease, but only in the CA1-2 hippocampal fields. However, due to the localization of ASIC1a and ASIC2b in the same hippocampal cellular subtypes, the decrease in both ASIC subtype levels may have a larger impact on the expression of native ASIC receptors in CA1-2 with respect to the other hippocampal fields. Furthermore, we have demonstrated that these changes are not due to the loss of neurons expressing ASIC mRNA, since animals having received an effective neuroprotective treatment produced similar results. Time-course analysis indicates that the downregulation of ASIC2b mRNA levels occurs quickly after the beginning of pilocarpine-induced neuronal hyperactivity. This finding suggests that the ASIC2b downregulation is related more to neuronal activity than to cell damage, which develops only after longer time intervals. Moreover, these changes appear to be long-lasting, persisting several hours after the initial neuronal activation, as evidenced by the depressed mRNA levels measured 24 h after pilocarpine injection in the diazepam+pentobarbital-treated animals.

The pilocarpine model of SE has been largely used to analyze changes in gene expression caused by seizures. Growth factor genes are massively stimulated following pilocarpine-induced convulsions, as reported for brain-derived neurotrophic factor, nerve growth factor, acidic and basic fibroblast growth factors, and glia-derived neurotrophic factor, but not for neurotrophin-3, which appears to be downregulated (reviewed in Gall et al., 1997). Growth factor receptors such as trkB are also upregulated, suggesting that in some cases there can be a stimulation of downstream

**FIG. 8.** Semiquantitative microdensitometry of in situ hybridization for ASIC1a, ASIC2a, and ASIC2b. Several groups of treatments were compared to one another: saline (SAL), pilocarpine at subconvulsive dose (P200, 200 mg/kg), pilocarpine at convulsive dose (P380, 380 mg/kg), pilocarpine at convulsive dose followed by combined diazepam (10 mg/kg) and pentobarbital (30 mg/kg) (P380/DZ+PB), or diazepam and pentobarbital combination alone (DZ+PB). Data are represented as optical density values (O.D., means ± SD). *P < 0.05, **P < 0.01, one-way analysis of variance followed by post hoc Dunnett’s test for multiple comparisons.
transductional pathways (Merlio et al., 1993; Schmidt-Kastner et al., 1996). These changes in growth factor synthesis have been viewed as the activation of local restorative mechanisms (Lindvall et al., 1994).

A downregulation of mRNA expression has been found for some excitatory channels in epilepsy models other than pilocarpine. In the hippocampal kindling paradigm, mRNA levels for the glutamate receptor subunits of the N-methyl-d-aspartate subtypes NR2a and NR2b have been found to decrease by around 50% specifically in dentate gyrus granule cells during the kindling procedure as well as in fully kindled rats (NR1 subunit) (Pratt et al., 1993). GluR1 and GluR2 AMPA receptor subunits have also been found to decrease to approximately 50% of the basal mRNA levels in cultured brain slices stimulated with picrotoxin (Gerfin-Moder et al., 1995) or in in vivo models (Prince et al., 1995). This phenomenon may represent a mechanism of neuroprotection during epilepsy (Gerfin-Moder et al., 1995). However, other investigators have proposed that the selective downregulation of GluR2 may be a critical event preceding neuronal cell death, because of the imbalance in the ratio between calcium impermeable (GluR2) and permeable (GluR1) channel subunits favoring the rise of intracellular calcium to toxic levels (Grooms et al., 2000). Although the involvement of ASICs in neurodegenerative events has been suggested, we have not observed any evident relation between ASIC expression during epilepsy and cell damage. In fact, downregulation of the ASIC2b subunit was found both in highly damaged areas such as CA3 and dentate hilus and in preserved regions such as the dentate gyrus (Liu et al., 1994; Fujikawa, 1996) and regardless of the neuroprotective diazepam + pentobarbital treatment. By contrast, GluR2 subunits were not downregulated but actually increased in neurons of dentate gyrus resistant to kainic acid-induced lesion (Grooms et al., 2000).

More recently it has been shown that pilocarpine-related seizure activity may cause changes more subtle than those related to neuronal death that could also be involved in the appearance of spontaneous recurrent seizures. Specifically, GABA<sub>α</sub> receptor subunits are differently expressed after SE, with increased mRNA levels of α4, β3, δ and ε subunits and decreased expression of α1 and β1 subunits, causing a shift of GABA<sub>α</sub> receptor composition toward a phenotype less sensitive to benzodiazepine stimulation but presenting enhanced GABA responsiveness (Brooks-Kayal et al., 1998). Because the native composition as well as the pharmacology of ASIC receptor is still not defined, we cannot draw any definite conclusion about functional changes in the ASIC receptor due to altered subunit expression. However, since it is possible that ASIC1a and ASIC2b might coassemble in vivo as in heterologous systems, the downregulation of ASIC1a and ASIC2b subunits in the CA1 field could lead to an overall blunted responsiveness to low pH, due to a decrease in receptor synthesis. Alternatively, in other hippocampal regions, the downregulation of ASIC2b subunits could result instead in changes of ASIC receptor properties, due to a modification of the

FIG. 9. Photomicrographs of the CA3 hippocampal field showing the lesion caused by pilocarpine-induced status epilepticus (SE). A shows a pilocarpine-treated rat in which SE has not been arrested. Damaged cells appear pinkish (arrows), due to the more intense eosin staining (acidophilia). B shows an animal in which seizures were stopped with diazepam (10 mg/kg) and pentobarbital (30 mg/kg) after 30 min of status epilepticus. Calibration bar, 50 μm.
ratio of expression of ASIC subunits. However, a functional interaction between ASIC1a and ASIC2b still remains to be demonstrated as previously shown for other ASIC subunit combinations (Lingueglia et al., 1997; Babinski et al., 2000). ASIC2b subunits have the ability to modulate ASIC functional properties (Lingueglia et al., 1997). This could mark them as potential targets for neuromodulatory substances whose function is to regulate the response of ASIC receptor to low pH. Evidence for the existence of modulatory neuropeptides active on various ASIC homomultimeric channels has been recently obtained (Askwith et al., 2000), and it has also been shown that substances able to modulate the ASIC channels in homomultimeric form can be even more powerful when acting on coassembled ASIC subunits (Babinski et al., 2000). Thus, the downregulation of ASIC2b mRNA levels reported here in the pilocarpine model of temporal lobe epilepsy suggests that selected factors released by overstimulated neurons could specifically recognize ASIC2b subunits and regulate the function of ASIC receptors in the epileptic hippocampus.

In conclusion, ASIC receptors appear to be an interesting novel class of excitatory channels with widespread distribution in the central nervous system, potentially involved in many sensory, motor, and visceral functions as well as higher brain functions, as suggested by the high levels of expression in cerebellum, limbic system, and cerebral cortex. The various ASIC subunits are differentially expressed on a regional basis and could thus coassemble into heteromeric ASIC receptors with different properties. In the hippocampus, ASIC1a and ASIC2b are localized in the same cellular types and could thus constitute heteromeric channels participating in normal and pathophysiological activities of this structure. The preferential downregulation of ASIC2b subunits in our model of epilepsy suggests that neuromodulatory substances might recognize ASIC receptors and regulate their function. Putative candidates for this role could be found among neurotransmitters or neuromodulators involved in limbic seizure activity.

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