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Polyamines Modulate AMPA Receptor–Dependent Synaptic Responses in Immature Layer V Pyramidal Neurons

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Shin, Jieun, Fran Shen, and John R. Huguenard. Polyamines modulate AMPA receptor-dependent synaptic responses in immature layer V pyramidal neurons. *J Neurophysiol* 93: 2634–2643, 2005. First published December 1, 2004; doi:10.1152/jn.01054.2004. α-Amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors (AMPARs) mediate the majority of fast excitation in the CNS. Receptors lacking GluR2 exhibit inward rectification and paired-pulse facilitation (PPF) due to polyamine (PA)-dependent block and unblock, respectively. In this study, we tested whether rectification and PPF in immature, but not mature, pyramidal neurons depend not only on the absence of functional GluR2 but also on the level of endogenous PAs. Whole cell recordings were obtained from layer V pyramidal neurons of P12–P14 or P16–P20 rats in the presence or absence of spermine in the pipette (50 μM). Isolated minimal excitatory synaptic responses were obtained, and paired (20 Hz) stimuli were used to investigate the rectification index (RI) and paired-pulse ratio (PPR). Spermine and its synthetic enzyme, ornithine decarboxylase (ODC), expression was examined using immunostaining and Western blot, respectively. At the immature stage (<P15) inclusion of intracellular spermine increased rectification and PPF for evoked excitatory postsynaptic currents (EPSCs) but had little or no effect on either measure in more mature (P16–P20) pyramidal neurons. Depletion of PAs reduced rectification suggesting that endogenous PAs play a critical role in functional regulation of AMPARs. Spermine immunoreactivity and ODC expression in immature rat neocortex (<P15) were greater than more mature tissue by ~20 and 60%, respectively. These results provide further support for the idea that excitatory synapses on immature neocortical pyramidal neurons ubiquitously contain AMPA receptors lacking the GluR2 subunit and that the level of endogenous PAs plays an important role in modulating AMPAR-dependent neurotransmission.

INTRODUCTION

α-Amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors (AMPARs) are ionotropic glutamate receptors that are largely responsible for fast excitation in CNS and likely play a key role in generation and spread of cortical seizure activity. AMPARs are heteromeric glutamate receptors, consisting of GluR1, 2, 3, and 4 subunits (Hollmann and Heinemann 1994; Sommer et al. 1991). AMPARs assembled without GluR2 are Ca^{2+} permeable and inwardly rectifying (Geiger et al. 1995 1995; Gu et al. 1996; Jonas and Burnashev 1995; Washburn et al. 1997). Inward rectification occurs by voltage-dependent blockade by polyamines (PAs), primarily spermine (Kamboj et al. 1995; Koh et al. 1995; Panchenko et al. 1999; Paschen 1992; Paschen et al. 1991; Smith and Chesler 1999; Washburn et al. 1996).

Spermine is a commonly expressed PA in many CNS neurons (Pellegrini-Giampietro et al. 2003). PAs are present in almost all cells and are implicated in physiological roles such as regulation of cell division and protein synthesis. Proliferating and differentiating cells express high PA levels, and PAs have specific functions in the nervous system (Soluet and Rivest 2003). They are the most cationic molecules in cells and thus strongly interact with physiologic anions like DNA, RNA, and membrane phospholipids (Thomas et al. 2002). The ionic and hydrophobic interaction provides a powerful means to regulate ion channel activity and receptor function. For instance, spermine has been shown to interfere with the phosphoinositide/Ca^{2+}-signaling pathway (Hughes et al. 1994; Porter and Bergeron 1983), since it competes with Ca^{2+} and is present in the intracellular medium (Coburn et al. 2002; Ozaki et al. 2000).

Recent studies have shown that synaptic responses mediated by AMPARs lacking GluR2 exhibit paired-pulse facilitation (PPF) via a voltage- and use-dependent PA unblock (Rozov and Burnashev 1999), and immature pyramidal neurons express low levels of functional synaptic GluR2 (Kumar et al. 2002). Therefore there is interest in the role of PAs in the functional regulation of AMPARs during development. Based on previous reports, we hypothesized that introduction of exogenous PAs via a whole cell recording pipette would increase intracellular PA concentration and enhance inward rectification and use-dependent PA-dependent unblock of GluR2-deficient synaptic AMPARs. Immature (<P15) rat neocortical pyramidal neurons lack GluR2; therefore synaptic AMPARs are Ca^{2+} permeable and inward rectifying. In contrast, older rats express high levels of synaptic GluR2 (Kumar et al. 2002), which results in blockade of Ca^{2+} influx via an electrostatic hindrance from an arginine within the pore region of the channel (Burnashev et al. 1992; Hollmann et al. 1991). This study was designed to examine plastic mechanisms mediated by known endogenous modulators of AMPARs—the PAs.

METHODS

Slice preparation

Slice preparation and electrophysiology have been previously described (Kumar et al. 2002). Briefly, Sprague-Dawley rats (P12–P20) were anesthetized with pentobarbital (50 mg/kg) and decapitated. Cortical slices from the brain were cut coronally on a vibratome in a 4°C sucrose solution containing (in mM) 234 sucrose, 11 glucose, 24

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$NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, and 0.5 $CaCl_2$, equilibrated with a 95%-5% mixture of O_2 and CO_2. The prepared slices were incubated in oxygenated artificial cerebrospinal fluid (ACSF; in mM: 126 NaCl, 26 $NaHCO_3$, 2.5 KCl, 1.25 NaH_3PO_4, 2 $CaCl_2$, 2 $MgCl_2$, and 10 glucose, pH 7.4) at 32°C for 1 h and subsequently in the same solution at room temperature.

Recently, P12–P14 (low GluR2 expression) and P16–P20 (high GluR2; Kumar et al. 2002) have been defined as the immature and mature developmental stages of AMPARs.

Electrophysiology

Recordings were made from layer V pyramidal neurons from rat neocortical brain slices (300-μM coronal sections) at two developmental stages: P12–P14 or P16–P20. Recording electrodes (1.2- to 2-μm tip diam, 3–6 MΩ) were filled with internal solution composed of (in mM) 120 cesium gluconate, 1 $MgCl_2$, 1 $CaCl_2$, 11 KCl, 10 HEPES, 2 NaATP, 0.3 NaGTP, 1 *N*-(2,6-dimethylphenylcarbamoylmethyl) triethylammonium bromide (QX-314), and 11 EGTA, pH 7.3, corrected to 290 mOsm. Membrane voltages were not corrected for liquid junction potential. Drugs and chemicals were applied through the perfusate that was continuously oxygenated with 95% O_2-5% CO_2. Recordings were performed at room temperature. Whole cell voltage-clamp recording was used to record EPSCs evoked by paired pulse repetitive stimuli at intervals of 50 ms. Spermine (50 μM) was either included or excluded from the pipette solution. Concentric bipolar electrodes with 75 μm OD (CB-XRC75, Frederick Haer and Co.) were positioned intracortically in close proximity to the recorded neuron. Constant current pulses, 20–100 μs in duration and 100–500 μA in amplitude, were applied at low frequencies (0.1–0.3 Hz). Minimally evoked synaptic responses were obtained as follows: stepwise increases in stimulus duration were applied until postsynaptic responses could be just detected, and then stimulus duration was held constant at ~1.2 times the threshold value throughout the remainder of the experiment (thresholds were characterized by a large proportion of failures; Dobrunz and Stevens 1997). Recordings were made with a Multiclamp 700A amplifier (Axon Instruments, Union City, CA), filtered at 1–2 kHz, and digitized at 10 kHz using pClamp software (Axon Instruments). Series resistance was 8–10 MΩ, and those experiments in which this parameter changed by >20% were rejected. Series resistance compensation was not ordinarily used. Excitatory postsynapatic potentials (EPSPs) were obtained under similar stimulus conditions to those used for excitatory postsynpatic current (EPSC) measurement, i.e., minimal stimulation was used and pairs of stimulus pulses were delivered at 20 Hz. Current-clamp recordings were performed with an intracellular solution similar to that used in voltage clamp, but with potassium gluconate replacing cesium gluconate.

To isolate AMPAR responses, a cocktail solution containing 50 μM PTX, 100 μM APV, and 0.1 μM NBQX was applied in bath solution (Kumar et al. 2001). Rectification index (RI) was determined as the slope of the *I-V* curve at positive potential (40–0 mV) divided by the slope of the *I-V* curve at negative potential (0 to −50 mV). Each of these regions of the *I-V* curve was sufficiently linear to allow accurate measurement of the RI. Specifically, the correlation coefficients, Pearson's *r*, were >0.88 at positive potentials ($P < 0.05$) and >0.85 at negative potentials ($P < 0.05$). Paired pulse ratio (PPR) was defined as the ratio of the peak amplitudes of the second and first EPSC in each pair. EPSP amplitudes were determined by measuring the voltage difference between the membrane potential 1 ms prior to each stimulus and that at the peak of the corresponding synaptic response.

Immunohistochemistry

P12–P14 and P16–P20 Wistar rats were anesthetized and transcardially perfused a fixative composed of 4% paraformaldehyde and 0.5% glutaraldehyde. The isolated brain slices were cryoprotected, by immersion in 30% sucrose until they sunk, and were resectioned at 35 μm with a freezing microtome (HM 400, Microm).

Immunocytochemical labeling for spermine was obtained via standard diaminobenzidine (DAB) immunoperoxidase protocols (Laube and Veh 1997). Briefly, each free-floating section obtained from P12–P14 rats and P16–P20 rats were placed together in one incubation well for the entire experiment to make sure the same treatment. Sections were sequentially exposed to polyclonal spermine antibody (Chemicon International, Temecular, CA) for 48 h (1:1,000, 4°C). After rinsing twice for 10 min in PBS, the sections were incubated with a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) followed by ABC reagent employed for the avidin-biotin staining technique (Vectastain Elite Kit, Vector Laboratories), and visualized with DAB (Sigma) as the chromogen. Paired sections were then mounted on gelatin-coated slides, air-dried, dehydrated with ascending series of ethanol, and coverslipped with DPX mounting media (Aldrich Chemical Co., Milwaukee, WI). Images were taken from the layer V cortex at about 3 mm from midline at the same hemisphere side of each paired sections, and the area of each image was 350 × 280 μm using a ×40 objective lens. Spermine immunoreactivity was determined by measuring the mean intensity of paired images of the two age groups. Three pairs of nonadjacent sections from each animal were used for image analysis.

Western blot

Cortical slices were prepared from the brain of either P12–P14 or P16–P20 rats. Cortical regions were isolated and homogenized in tissue protein extraction reagent (T-PER, Pierce, Rockford, IL) containing EDTA-free protease inhibitor cocktail (Pierce). Samples were centrifuged at 10,000*g* for 5 min, supernatants were separated in duplicate on 10% polyacrylamide gels, and proteins were analyzed by Western blotting using antibodies against ornithine decarboxylase (ODC; Sigma; 1:5,000 dilution) and β-actin (Sigma; 1:10,000 dilution). Immunoreactivity was detected by enhanced chemiluminescence. We measured the amount of ODC and β-actin immunoreactivity in each sample as intensity values of the corresponding band. The ratio of ODC to β-actin was expressed relative to the mean value measured in the same lane of final film.

Materials

2,3-Dihydro-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX, diluted in dimethylsulfoxide, <0.1% final concentration), spermine tetrachloride (spermine), and picrotoxin (PTX) were purchased from Sigma, D(−)-2-amino-5-phosphonopentanoic acid (D-AP5, diluted in 0.1 N NaOH) was from Tocris, and α-difluoromethylornithine (DFMO) was from Calbiochem.

RESULTS

Effect of exogenous PAs on rectification

Inward rectification is a characteristic property of AMPAR responses in nonpyramidal neurons and immature pyramidal neurons, and this is correlated with a deficiency in GluR2 subunits (Jonas et al. 1994; Kumar et al. 2002; Washburn et al. 1997). This study investigated the influence of exogenous and endogenous PAs on inward rectification in neocortical pyramidal neurons as a function of age. We studied two distinct developmental stages, P12–P14 and P16–P20, of experimental rats. Evoked AMPAR-dependent synaptic responses in neocortical pyramidal neurons from P12–P14 rats displayed a rectification index of 0.54 ± 0.04, even in the absence of exogenous PAs (Fig. 1*A*, $n = 8$, see METHODS for analysis of RI;

values <1 represent inward rectification). This value is consistent with our earlier findings of inward rectification, lower expression of somatic GluR2 immunoreactivity and higher synaptic AMPAR Ca^{2+} permeability at this stage (Kumar et al. 2002). Additions of spermine (50 μM) to the intracellular solution resulted in much stronger inward rectification with a resultant RI of 0.36 ± 0.04 (n = 7, Fig. 1A, +spermine vs. –spermine, $P < 0.01$). These data suggest that significant endogenous PAs remain at excitatory synaptic sites in neocortical pyramidal neurons during whole cell recording—i.e., they are incompletely dialyzed by the spermine-free patch pipette contents. This contrasts with results obtained with either somatic or cell-free recording in other cell types, where rectification of AMPAR responses was shown to wash out during maintained recordings (Donevan and Rogawski 1995; Isa et al. 1995; Kamboj et al. 1995). Even though dialysis of synaptic PAs is apparently incomplete, the increase in rectification produced by spermine indicates that synaptic PA levels can be augmented from exogenous sources (see DISCUSSION).

In contrast, synaptic responses in pyramidal neurons from P16–P19 rats exhibited outward rectification (Fig. 1*B*), consistent with prominent GluR2 expression (Higuchi et al. 1993; Jonas and Sakmann 1992; McBain and Dingledine 1993). In this age group, inclusion of spermine in the pipette had little effect on rectification—RI was 1.13 ± 0.47 in the absence of spermine and 1.13 ± 0.35 in its presence (n = 6, $P > 0.1$). The *I-V* relationship for AMPAR-dependent EPSCs with PA-containing intracellular solution was not significantly different from that obtained with PA-free pipette solution (Fig. 1*B*), indicating that PA-augmented rectification is dependent on the absence of GluR2.

To determine the generality of these findings with respect to input specificity, we recorded spontaneous EPSCs (sEPSCs), presumably arising from a variety of presynaptic sources, under identical conditions as those described above for intracortically evoked EPSCs. sEPSCs were analyzed and compared at holding potentials of +40 and −60 mV. In P13 neocortical pyramidal neurons, spermine increased rectification, causing a change in RI from 0.83 ± 0.06 (n = 5) to 0.62 ± 0.10 (n = 6, $P < 0.05$, Fig. 2). However, in neurons from P17 rats, sEPSCs were outwardly rectifying, with RIs that were comparable in both PA-free and PA-containing conditions (1.29 ± 0.05, n = 5 and 1.18 ± 0.10, n = 6). The averaged peak amplitude of sEPSCs measured at +40 and −60 mV in the presence of PA was similar to the cortically evoked EPSC at the same holding potentials, which is consistent with the idea that the evoked EPSCs were "minimal," i.e., arising from a single presynaptic fiber.

Thus we observed that both spontaneous and evoked AMPAR-mediated EPSCs in the younger (<P15) animals display age-dependent differences in rectification compared with the older animals, suggesting discrete physiological properties of the underlying receptors at these ages. Furthermore, the effects

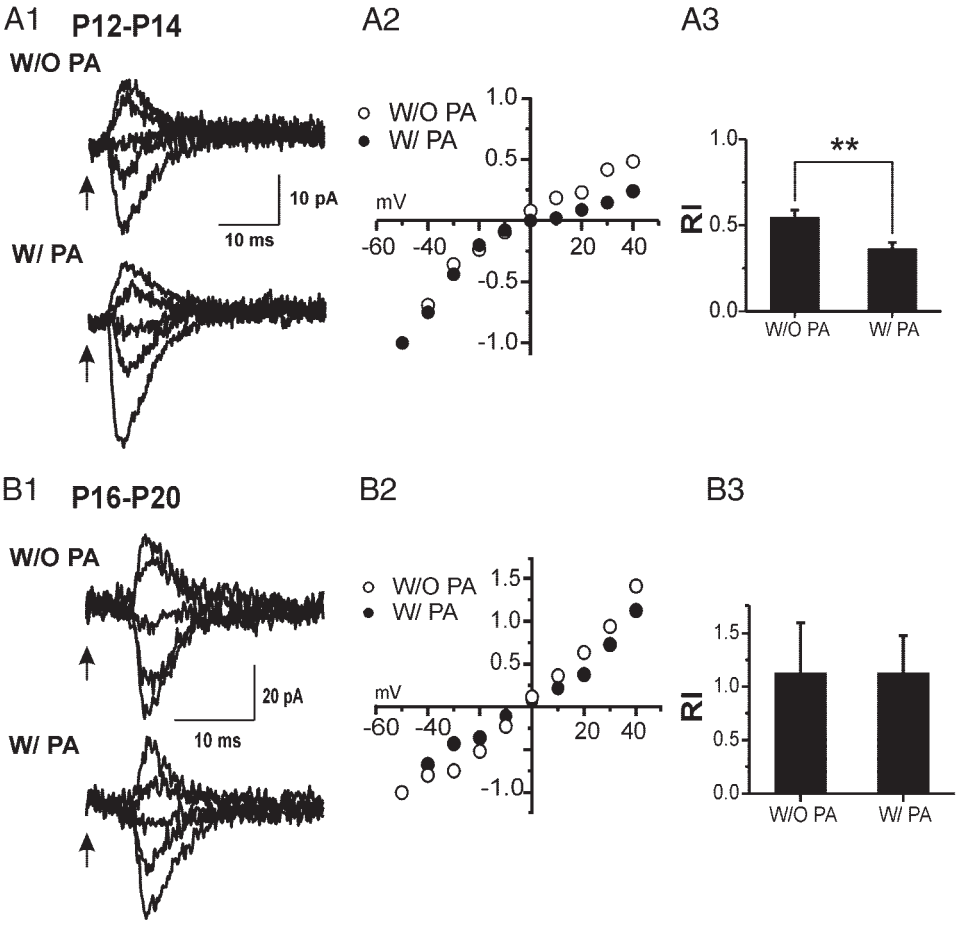

FIG. 1. Exogenous polyamine (PA) causes increased inward rectification in immature but not in mature layer V synapse. *A1*: representative excitatory postsynpatic currents (EPSCs) recorded from a P12–P14 rat neocortical pyramidal neuron at potentials of −50, −30, 0, +20, and +40 mV. *A2*: *I-V* curves for minimally evoked synaptic currents in 2 groups of neurons (i.e., either with or without PA in the pipette) at various holding potentials. *I-V* curves, normalized to EPSC amplitude at −50 mV, show increased rectification in the presence of PA. *A3*: PA-dependent effects on rectification indices (Ris) in immature neurons (n = 12, 9; $^{**}P < 0.01$). *B1* and *B2*: EPSCs in neurons from P16–P20 rats were obtained under equivalent methods to those used in *A*. *A2* and *B2*: normalized *I-V* relationships of pooled data. Each point on the plots (○, recording without PA; ●, recording with PA) represents an ensemble average of 7 experiments. *B3*: PA had little effect on RIs in older neurons (n = 5, 8; $P > 0.05$). Error bars indicate SE.

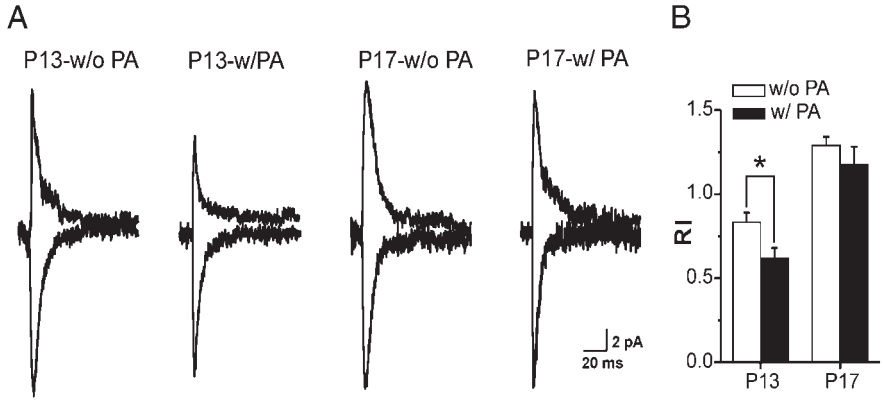

FIG. 2. Rectification of spontaneous EPSCs (sEPSC) in immature and mature rats. Representative traces are averages of all the successfully isolated sEPSCs occurring at holding potentials of +40 or −60 mV. *A*: averaged sEPSC at 40 (*top*) and −60 mV (*bottom*) in example layer V pyramidal neurons obtained from P13 or P17 rats. *B*: RIs at 2 ages obtained in the presence or absence of intracellular spermine. Exogenous PAs decreased RI only in neurons from younger (P13) rats ($*P < 0.05$, $n = 8$). Each averaged EPSC was obtained from ≥50 individual sEPSCs. sEPSC RI is the ratio of amplitude at 40 mV divided by that measured at −60 mV.

of exogenous PAs on both types of AMPAR-dependent signaling support a difference in PA sensitivity.

Synaptic site of PA effects

To determine whether the effect of PAs on AMPAR function was pre- or postsynaptic, we used a paired pulse protocol to test for changes in a postsynaptic measure, RI. A decrease in inward rectification (a higher value for RI) for the second compared with the first EPSC would suggest PA unblocking as has been shown in interneurons that express GluR2-deficient synaptic AMPARs (Rozov and Burnashev 1999). *I-V* relationships in P13 neurons were determined for each of a pair of synaptically evoked responses (50-Hz stimulation frequency). In general, there was a decrease in rectification for the second EPSC compared with the first, whether or not spermine was included in the pipette.

Under PA-free recording conditions, there was a modest decrease in rectification between the first and second EPSCs (RI 1st: 0.60 ± 0.03, RI 2nd: 0.73 ± 0.05, $n = 4$, $P < 0.05$). Examination of the original traces obtained during recordings with PA-containing pipettes revealed a progressive increase in second responses at positive potential and decrease at negative potential compared with the first. The *I-V* relationship for the first EPSC in the presence of PA showed robust inward rectification, as shown in Fig. 1 (in immature rats). However, the *I-V* relationship for the second EPSC deviated from that for the first EPSCs, especially at positive potentials, consistent with activity dependent relief from PA block at postsynaptic site. RIs closer to one reflect a reduced rectification. In the presence of PA, RIs were smaller (i.e., greater rectification), and as in the PA-free condition, there was an increase in RI between the first and second EPSC (RI 1st: 0.34 ± 0.06 and RI 2nd: 0.57 ± 0.04, $P < 0.01$, $n = 5$).

PPF was consistently observed in recordings with spermine-containing intracellular solutions (Fig. 3). Larger amplitude of the second compared with the first EPSC in the presence of

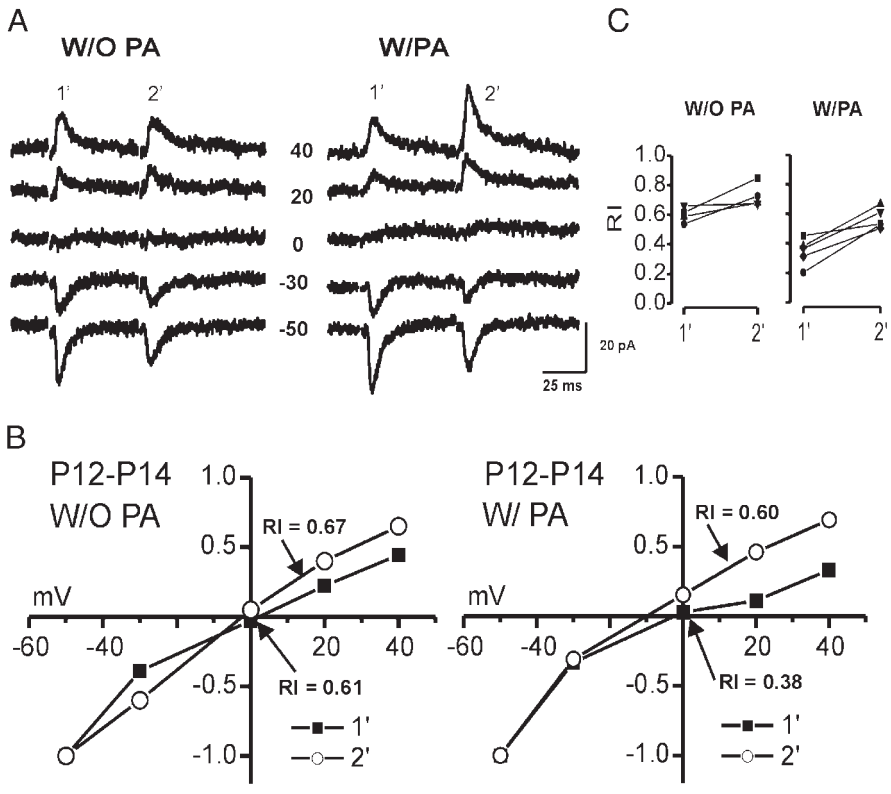

FIG. 3. Postsynaptic synaptic plasticity mediated by PA unblocking. *A*: representative traces are averages of >3 consecutive trials at various holding potentials of 40, 20, 0, −30, and −50 mV. Paired responses (50-ms interpulse interval) in a young (P13) neuron obtained in the absence (*left*) or presence (*right*; 50 μM spermine). *B*: *I-V* curves for the 1st (1′) and 2nd (2′) EPSC in each pair. Note the decreased rectification in the 2nd compared with the 1st EPSC (*left*). This difference is magnified in the presence of exogenous PAs (*right*) *C*: RIs, derived from *I-V* curves for the 1st and 2nd EPSCs. Each symbol (with line connector) indicates RIs of paired α-Amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor (AMPAR)-dependent EPSCs obtained from *I-V* curves in individual neurons either with PA (spermine, W/ PA: *right*) or without (W/O PA: *left*) in the patch pipette. PAs increases rectification, but this is relieved in the 2nd pulse (2′; paired *t*-test, $P < 0.01$). Note that the RI of the unblocked 2′ response obtained with spermine is similar to the 1′ response obtained without exogenous PAs.

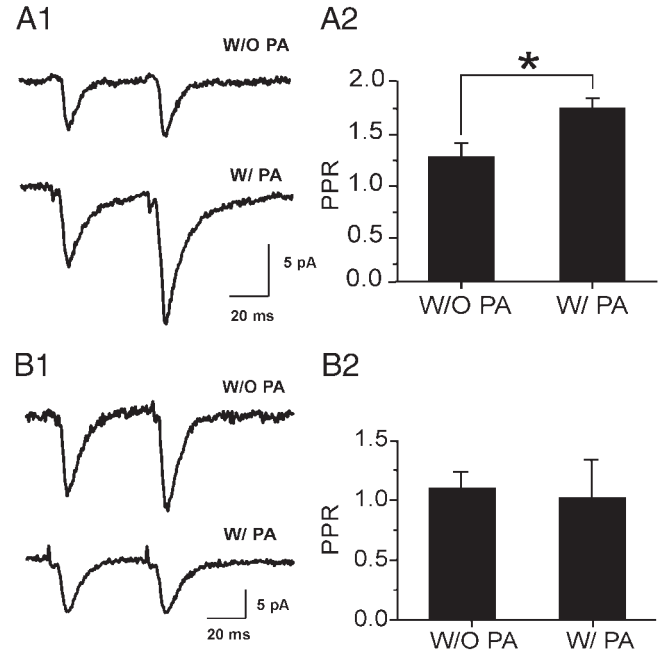

FIG. 4. PAs induce paired-pulse facilitation (PPF), but only in P12–P14 rats. *A1*: representative traces are averages of >50 consecutive responses. Paired stimuli separated by 50 ms (20 Hz) in P12–P14 rats and P16–P20 rats. *A2*: paired-pulse ratio (PPR, value obtained by dividing average amplitude of the 2nd response by that of the 1st) was increased by PA ($n = 12$, $^*P < 0.05$) in P12–P14 rats. *B1*: paired stimuli provided traces in P16–P19 rats. *B2*: PPR was invariant in presence of PA in the P16–P19 rats ($n = 12$). In all cases, holding potential was −60 mV.

spermine indicated that PA-dependent facilitation provides an entirely postsynaptic mechanism of dynamic regulation of synaptic gain that may determine target-cell specific differences in synaptic transmission in neuronal circuits.

Dynamic PA-dependent changes in PPR

To determine whether PAs might exert their effects on short-term synaptic plasticity through a mechanism independent of (age-dependent) GluR2 interaction, we determined the PPR of AMPAR-dependent EPSCs in two developmental age groups where we expected differences in synaptic GluR2 expression (Kumar et al. 2002). Short-term presynaptic change such as depression or facilitation should be dependent on previous release (Debanne et al. 1996). Thus in a paired-pulse protocol, the ratio between the mean amplitudes of the second EPSC and the first EPSC (PPR) is inversely proportional to the initial release probability (Dobrunz and Stevens 1997). Inclusion of intracellular spermine increased PPR in immature (<P15) neurons (from 1.21 ± 0.11 to 1.68 ± 0.09, $n = 7$, $P < 0.05$), but had little or no effect on PPR (1.09 ± 0.14 to 1.01 ± 0.32, $n = 5$, not significant) in more mature (P16–P20) pyramidal neurons (Fig. 4).

Consequently, to test dynamic PA regulation of GluRs in P12–P14 rats, we examined time-dependent changes in PPRs at 2, 5, and 20 min after establishing whole cell recordings. In recordings with spermine, surprisingly, the amplitude of the second EPSC became higher than that of the first in a time-dependent manner (Fig. 5*A*). Figure 5*B* shows that PPR was significantly increased over time when in PA recordings (PPRs at 2, 5, and 20 min are 1.18 ± 0.09, 1.27 ± 0.05, and 1.57 ± 0.07, respectively, $n = 8$), but not in PA-free recordings. Therefore the PA-dependent changes in PPF strongly support a role in postsynaptic regulation of AMPARs. For the first response in each pair, the addition of PAs produced an increase in AMPAR-mediated rectification in a time-dependent manner ($RI_{2min} = 0.33$, $RI_{5min} = 0.25$, $RI_{20min} = 0.23$, Fig. 5*C1*). In contrast, recordings made with PA-free solutions failed to show increased rectification but

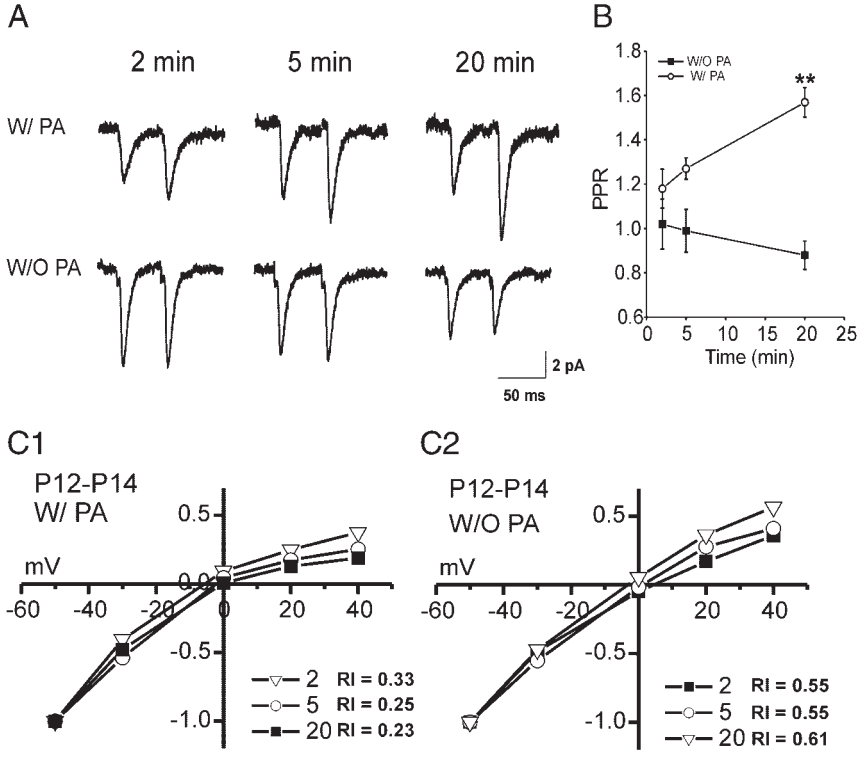

FIG. 5. PAs cause dynamic change in PPR in immature rats. *A1*: representative traces are averages of >40 consecutive responses at a holding potential of −60 mV. Paired pulse responses were measured at 2, 5, and 20 min after establishing the whole cell patch clamp using the same protocol used in Fig. 4. *B*: mean PPR recorded at −60 mV in PA-free (W/O PA, filled symbols, $n = 5$) and PA-containing conditions (W/ PA, open symbols, $n = 7$) in P12–P14 rats. Each symbol represents means ± SE. PPR significantly increased in 20 min after the recording but only in the presence of spermine ($^{**}P < 0.01$). *C1*: Example *I-V* curves showing the progressive increase in rectification concomitant with increased PPR evident in *A* (*top*). *C2*: *I-V* curves showing the progressive decrease in rectification concomitant with decreased PPR evident in *A* (*bottom*).

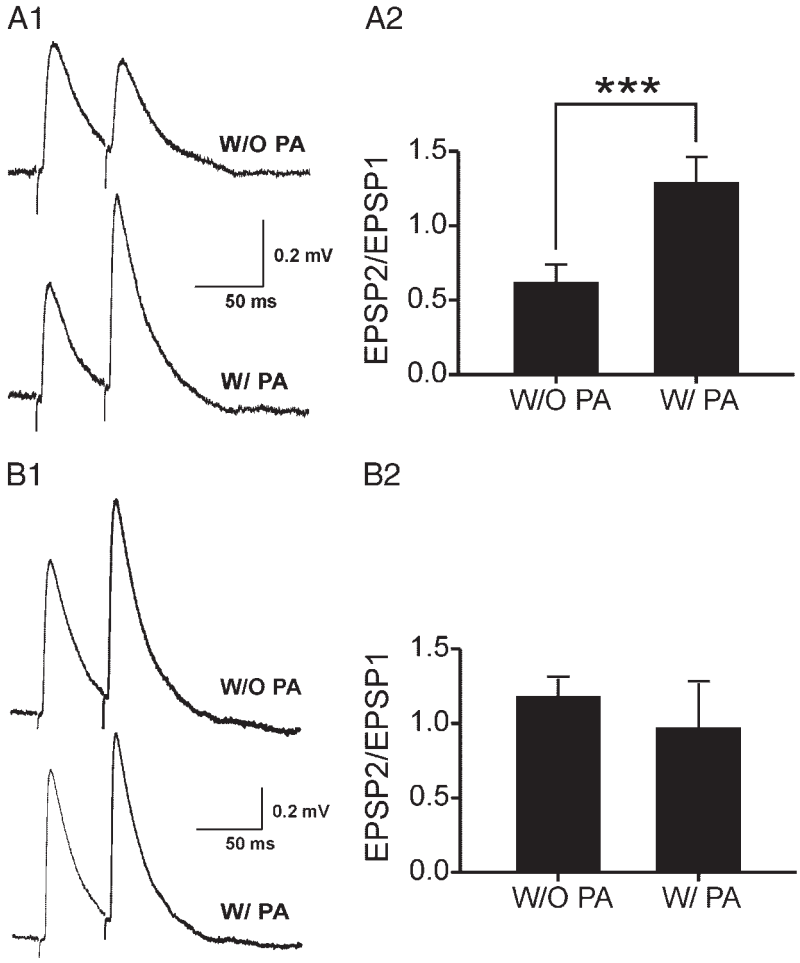

FIG. 6. PA-dependent facilitation of AMPAR excitatory postsynpatic potentials (EPSPs). *A1*: representative traces are averages of >60 consecutive synaptic responses at resting membrane potential (V_m, −58 mV, *top*; −54 mV, *bottom*) in P12–P14 rats layer V neocortical pyramidal neurons. EPSPs were evoked by paired (20 Hz) stimuli in the absence (W/O PA, *top*) or presence (W/ PA, *bottom*) of exogenous pipette spermine (50 μM). *A2*: PPR, obtained by dividing the average amplitude of the 2nd EPSP (EPSP2) by that of the 1st (EPSP1), was increased by PA ($n = 6, 6$, $^{***}P < 0.001$) in P12–P14 rats. *B1*: responses to paired stimuli in P16 layer V pyramidal neurons (V_m, −54 mV, *top*; −56 mV, *bottom*). *B2*: PPR was not affected by PA in P16–P19 rats ($P > 0.05$, $n = 4, 4$).

rather showed a time-dependent decrease in the rectification ($RI_{2min} = 0.55$, $RI_{5min} = 0.55$, $RI_{20min} = 0.61$, Fig. 5*C2*). These results further support that PA levels dynamically regulate synaptic AMPAR function.

Because PA unblock is both voltage- and use-dependent, we performed a set of experiments to determine the degree of unblocking that would occur under physiological conditions, i.e., in current-clamp mode in which membrane potential is allowed to vary during synaptic activation. Under these conditions, in immature (P12–P14) neurons, exogenous intracellular spermine elicited PPF of EPSPs compared with recordings obtained in the absence of exogenous spermine (Fig. 6, *A1* and *A2*). In contrast, in mature pyramidal neurons (P16–P18), there was no effect of exogenous spermine on PPF (Fig. 6, *B1* and *B2*).

Function of endogenous PA

Given the striking difference in (PA-dependent) rectification of AMPAR-dependent EPSCs observed in P12–P14 neurons compared with responses in P16–P20 rats (Fig. 1*A*), we hypothesized that physiological regulation of endogenous PA would functionally modify AMPARs. To address this, we assayed rectification after spermine depletion via a 2-h incubation with diethylspermine (DES, 1 mM), a spermine analogue and an inhibitor of PA synthesis (Vertino et al. 1991). Depletion of spermine resulted in a reduced inward rectification; the RI was increased after DES treatment to 0.93 ± 0.12 ($n = 6$) from a control level of 0.56 ± 0.10 ($n = 7$, tandem control treated slices; data not shown). Additionally, DFMO (1 mM) an inhibitor of ODC, a primary biosynthetic enzyme for PAs, produced a decrease in inward rectification of synaptic AMPARs (Fig. 7; RI: control = 0.54 ± 0.04, $n = 5$; DFMO = 0.94 ± 0.11, $n = 6$, $P < 0.01$), indicating that, in immature layer V pyramidal neurons, constituitive ODC activity generates levels of PAs sufficient to modulate synaptic AMPAR function. This endogenous source of PAs may explain the lack of complete washout of inward rectification seen during whole cell recordings made without spermine in the pipette (Fig. 1*A*, cf. Kumar et al. 2002).

Age-dependent regulation of PA metabolism

The previous electrophysiological results suggested that not only exogenous PAs but also endogenous PAs were contributing to dynamic inward rectification of AMPARs in neocortical pyramidal neurons from immature rats. Accordingly, we expected that spermine might be up-regulated in P12–P14 rats compared with levels present in P16–P20 rats. Spermine has been shown to be distributed in both cell body and membrane of neurons as well as in the neuropil of neocortex (Gilad and Gilad 2002). To estimate spermine content in neocortical pyramidal neurons, we examined spermine-like immunoreactivity in cortex from P12–P14 rats. We also used Western blot

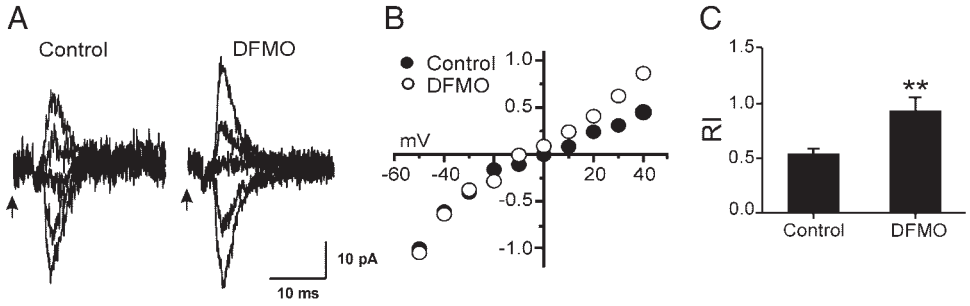

FIG. 7. α-Difluoromethylornithine (DFMO) inhibits inward rectification induced by endogenous PA. Every symbol shows averages of >3 consecutive responses. *A*: paired stimuli separated by 50-ms interval in a layer V pyramidal neuron from P13 rat. *B*: *I-V* characteristic of AMPAR-dependent synaptic current in DFMO-treated slices from P12–P14 rat. Before recording, brain slices were incubated in either DFMO (1 mM) containing artificial cerebrospinal fluid (ACSF; W/ DFMO) or DFMO-free ACSF for 2 h. *C*: Alterations in RI by DFMO (**$P < 0.01$).

to examine expression of ODC to address how PA metabolism might be differentially regulated in the different age groups. Tissue sections from P12–P14 rats showed more intense spermine-like immunostaining than those from P16–P20 rats. Spermine immunoreactivity appeared to be less intense in perineuronal region where the glial cell and neuropil are present (Gilad and Gilad 2002) in older (P20) rats compared with those from younger rats. Overall, taking into account neuronal staining, there was a 20% greater expression in P13 cortex compared P20 cortex (Fig. 8*A*, $n = 4$ animals each, $P < 0.05$). Consistent with increased levels of spermine at that stage, ODC expression was 1.6 ± 0.11 times higher in the younger compared with older cortex (Fig. 8*B*, $n = 4$ animals each, $P < 0.05$).

DISCUSSION

In this study, three properties expected for GluR2-deficient receptors were found using electrophysiological approaches in immature neurons: *1*) a RI < 1, indicating strong inward rectification; *2*) an increase in the rectification on addition of spermine; and *3*) an increase in PPR produced by spermine. We also found that exogenous PAs augment inward rectification and PPR only in the immature pyramidal neurons. Our results further support previous reports that application of either intracellular or extracellular PAs influence EPSCs derived from GluR2 deficient AMPAR in immature neocortical neurons (Kumar et al. 2002). Although GluR2-deficient AMPARs are unique in their selective block by external and internal PAs (Washburn et al. 1997), the potential influence of physiological PAs on immature synaptic AMPARs has not been established in a more functional way.

Accordingly, we investigated the effect of endogenous PAs using inhibitors of PA metabolism. As seen from the result shown in Fig. 7, depletion of endogenous PAs prevented and reversed inward rectification of AMPARs in developing neurons. This observation provides convincing evidence for a pivotal role of endogenous PAs in regulating GluR2-lacking synaptic AMPA receptors and producing PPF. Variation in levels of endogenous PAs indirectly supports a role in modulation of rectification. We found that in immature cortex, when AMPAR-dependent responses display inward rectification, there is relatively higher PA content as measured by immunostaining intensity. In this study, we found that the younger rats (<P15) expressed higher brain levels of both spermine and its metabolic enzyme, ODC, than P16–P20 rats. These biochemical results suggest that age-dependent alteration in PAs reflect alterations in PA synthesis. Those physiological and anatomical results from PA depletion together suggest a direct role of PAs in regulating inward rectification of GluR2-lacking AMPARs.

Surprisingly, the addition of PA into the pipette solution resulted in an increase in the amplitude of the second in a pair of EPSCs at negative potentials (−60 mV, Fig. 5). The second response should activate AMPARs in a relatively unblocked

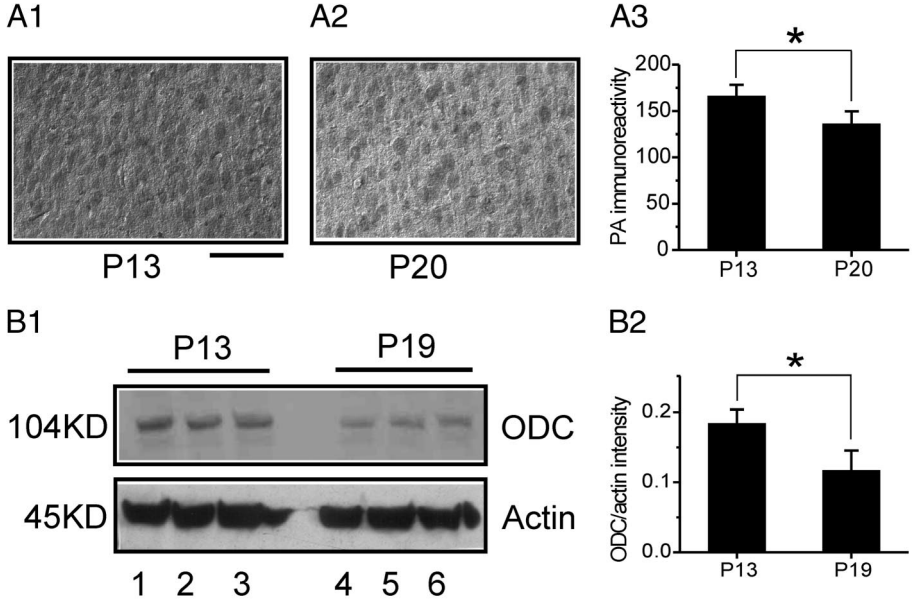

FIG. 8. Expression of PA is high in immature rat neocortex. *A1* and *A2*: spermine immunoreactivity in rat neocortical layer V in P13 (*A1*) and P20 (*A2*) rat brain slices. Note the increased spermine immunoreactivity in many neurons of the P13 rat. Scale bars; 100 μm. *A3*: optical density values for spermine immunoreactivity in equivalent regions of immature and mature slices ($n = 4$ rats each, 3 slices from each rat, *$P < 0.05$). *B1*: *top bands* represent ornithine decarboxylase (ODC) content and *bottom bands* represent β-actin for comparison as a housekeeping protein. Lanes 1 and 4 represent a band obtained from anterior region of cortical homogenates, lanes 2 and 5 from middle region of cortex, and lanes 3 and 6 from posterior. Lanes 1–3 are from cortical homogenates from P12–P14 rats; lanes 4–6 are from P16–P19 rats. *B2*: mean optical density of ODC bands normalized to β-actin. Western blots were visualized by SDS-PAGE and immunoblot (*$P < 0.05$).

state (Rozov and Burnashev 1999), one that is *less* dependent on intracellular PA levels. However, the second response was increased over time as the exogenous spermine was dialyzed into the neurons. One possible explanation is that PAs exert a nonspecific, possibly indirect, enhancing effect on AMPARs. This might result in an increased EPSC amplitude for both the first and second responses in each pair, with the first response then being decreased because of enhanced PA-dependent blocking. In any case, use-dependent unblocking should make the cell more responsive to repetitive synaptic inputs rather than to single stimuli, facilitating the detection of coincident activity when synaptic activity is especially intense.

In addition to AMPAR, PAs are known to interact with *N*-methyl-D-aspartate (NMDA) receptors (Benveniste and Mayer 1993, 1995; Williams 1997), voltage-dependent Ca^{2+} channels (Ferchmin et al. 1995), and inwardly rectifying potassium channels (Baukrowitz et al. 1998; Ishihara 1997; Ishihara et al. 2002; Oliver et al. 2000). In our experiments, we used APV, PTX, and Cs^+ to block NMDA and $GABA_A$ receptors, as well as K^+ channels, and thus our results likely reflect direct interactions with synaptic AMPAR channels.

Effect of Ca^{2+} on PA sensitivity to AMPAR

In all AMPAR subunits except GluR2, a critical pore-lining residue is arginine rather than glutamine. The positive charge resulting from the glutamine residue of even a single GluR2 subunit in the multimeric channel disrupts electrostatic interactions between the channel and both positively charged PAs and Ca^{2+} ions, and thus renders the channel Ca^{2+} permeable and rectifying, two common features of GluR2-lacking AMPARs (Burnashev et al. 1996; Hollmann et al. 1991).

It has recently been reported that, in immature neocortical pyramidal neurons, synaptic AMPARs are rectifying and Ca^{2+} permeable, and thus express low levels of GluR2 (Kumar et al. 2002). AMPARs in these immature rat neurons lack functional GluR2 subunit thus they become permeable to extracellular Ca^{2+} and can trigger intracellular Ca^{2+} dependent processes. Therefore PAs can regulate intracellular Ca^{2+} concentration via modulation of Ca^{2+}-permeable AMPA receptors, and it will be interesting to determine whether intracellular Ca^{2+} can regulate PA metabolism and function. Spermine has been found to be more effective in modulating PPR at lower rather than higher Ca^{2+} concentrations in CA1 hippocampal neurons (Ferchmin et al. 1995). Conversely, high Ca^{2+} prevents spermine from altering PPRs. It is interesting to speculate that part of the use-dependent unblocking of GluR2-deficient AMPARs by PA might be attributable to an increase in $[Ca^{2+}]_i$ via Ca^{2+}-permeable AMPA receptors. This increased Ca^{2+} might then alter spermine-sensitivity of the AMPARs and thus modulate PPR. Such a mechanism could contribute to greatly augmented EPSC responses during intense synaptic activity, such as occurs during epileptic seizures. The augmentation would be due to both facilitation resulting from reversal of polyamine block (Rozov and Burnashev 1999), but also indirectly through $[Ca^{2+}]_i$-dependent mechanisms.

PA metabolism during development

In addition to our earlier observation that immature rats (P12–P14) exhibited greater increase in inward rectification and PPR than mature rats (P16–P20), we showed a correlation between PA level and AMPAR activity by the showing that immature neurons express higher level of spermine than mature neurons.

In most cortical and hippocampal regions, spermine-like immunoreactivity in neurons was relatively weak, but the prominent localization of spermine in the pyramidal neurons of immature rats (P12–P14) might point to a functional role in PA and AMPA channel/receptor modification (data not shown). Consistent with an increase in the amount of spermine in the younger rats, ODC, a key metabolic enzyme for PA, was highly expressed in immature rats compared with more mature rats. These results indicated that PA metabolism was endogenously involved in altering AMPAR function. Accordingly, we propose two alternative mechanisms by which AMPARs are functionally regulated by PA in younger rats. On one hand, elevated PA level could prevent extreme increase in Ca^{2+} from the Ca^{2+}-permeable AMPA receptors. On the other hand, given the use-dependent PA unblock, PAs might be involved in augmenting synaptic strength.

The idea of PA regulation of AMPAR synaptic responses is consistent with the report of Aizenman et al. (2002) that visual activity regulates the synthesis of spermine and AMPA receptor currents in immature tectal neurons. Their studies support two possible mechanisms of the activity-dependent spermine block of Ca^{2+}-permeable AMPA receptors. First, they confirmed that elevated spermine levels could block AMPA-mediated responses and found that both spermine and visual stimulation reduce the amplitude of miniature EPSCs in tectal immature neurons, thus suggesting a possible neuroprotective role of PAs. Second, they revealed in agreement with our findings (Fig. 3) that the voltage-dependent block of immature AMPA receptors by spermine could be relieved by repetitive stimulation, leading to facilitation of synaptic transmission.

PAs seem to have an indispensable role in cell proliferation, because specific inhibition of their biosynthesis invariably halts the growth of mammalian cells. This likewise applies to high expressional level of PAs in developing neurons from P12–P14 rats (Janne et al. 2004). PAs are produced and metabolized by a group of enzymes. Of these, ODC is the critical rate-limiting step in PA metabolism. It seems to be a multifunctional protein and has the most rapid rate of synthesis and degradation among all mammalian enzymes (Casero et al. 2001; Wallace et al. 2003). Elevated ODC activity and expanded pools of the PAs are commonly associated with tumorigenesis and a role of oncogene-like protein has been assigned to ODC (Moshier et al. 1996; Seiler 2003).

Neuroprotective action of PA

Exogenous spermine and several PA derivatives have been proposed as neuroprotective agents due to their blocking of AMPA receptors (Jayakar and Dikshit 2004), which have prominently expressed in vulnerable regions following global ischemia and in neurodegenerative disorders (Kirby and Shaw 2004). In a rat suffering cerebral ischemia, spermine and spermidine were shown to be released from injured cells, and it has been suggested that they might ameliorate the symptoms of the ischemic episode by disrupting the toxic action of Ca^{2+} (Paschen et al. 1991, 1992). Early polyamine treatment enhances survival of sympathetic neurons after postnatal axonal

injury or immunosympathectomy induced by exposure to NGF in ganglionic nerves (Gilad and Gilad 2001).

Elevated ODC immunoreactivity has been observed in autopsied brain of patients with Alzheimer disease, suggesting an involvement of abnormal PA regulation in neurodegenerative processes (Choi et al. 2001; Morrison and Kish 1995; Morrison et al. 1998).

In addition, several lines of evidence suggest that polyamines may mediate or potentiate excitotoxic mechanisms responsible for neuronal damage during the hypoxic states (Zoli et al. 1993). Low GluR2 expression seems to play a major role in Ca^{2+}-dependent excitotoxicity and cell death (Ben-Ari et al. 1998).

Facilitation of inwardly rectifying AMPA receptors could be important during activity-dependent development of newly formed neural circuits. Consistent with this, voltage-dependent blocking of GluR2 deficient AMPAR in a PA-dependent manner, as shown in this study, might be one of the contributing factors diminishing synaptic excitability and balancing inhibitory signals, thus preventing seizures. These results thus have important implications regarding repetitive activation of neocortical networks both in the normal state and during developmental epileptic seizure disorders.

GRANTS

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