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Inositol Hexakisphosphate-Mediated Regulation of Glutamate Receptors in Rat Brain Sections

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ABSTRACT: D-myo-inositol 1,2,3,4,5,6-hexakisphosphate (InsP6), one of the most abundant inositol phosphates within cells, has been proposed to play a key role in vesicle trafficking and receptor compartmentalization. In the present study, we used in vitro receptor autoradiography, subcellular fractionation, and immunoblotting to investigate its effects on α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and N-methyl-D-aspartate (NMDA) receptors. Qualitative and quantitative analysis of ^{3}H-AMPA binding indicated that incubation of frozen-thawed brain sections with InsP6 at 35°C enhanced AMPA receptor binding in several brain regions, with maximal increases in the hippocampus and cerebellum. Moreover, saturation kinetics demonstrated that InsP6-induced augmentation of AMPA binding was due to an increment in the maximal number of AMPA binding sites. At the immunological level, Western blots performed on crude mitochondrial/synaptic (P2) fractions revealed that InsP6 (but not InsP5 and InsP3) treatment increased glutamate receptor (GluR)1 and GluR2 subunits of AMPA receptors, an effect that was associated with concomitant reductions in microsomal (P3) fractions. Interestingly, the InsP6-induced modulation of AMPA receptor binding was blocked at room temperature, and pretreatment with heparin also dampered its action on both AMPA receptor binding and GluR subunits. These effects of InsP6 appear to be specific to AMPA receptors, as neither ^{3}H-glutamate binding to NMDA receptors nor levels of NR1 and NR2A subunits in P2 and P3 fractions were affected. Taken together, our data strongly suggest that InsP6 specifically regulates AMPA receptor distribution, possibly through a clathrin-dependent process. *Hippocampus 2001;11:673–682.*

KEY WORDS: inositol phosphate; phytic acid; AMPA receptor; NMDA receptor; adaptin; clathrin

INTRODUCTION

D-myo-inositol 1,2,3,4,5,6-hexakisphosphate (InsP6), one of the most abundant inositol phosphates, is formed via complex pathways of inositol phosphate metabolism. In some cell types, its concentration is rapidly altered upon cell stimulation (Sasakawa et al., 1993, 1995), and experimental evidence indicates that it is involved in the control of several cellular functions. For instance, InsP6 has been shown to reduce light-induced desensitization of rhodopsin (Palczewski et al., 1992), presumably through direct binding to arrestin (Palczewski et al., 1991), a specialized class of regulatory proteins. In the brain, a number of high-affinity binding sites for InsP6 have been found (Hawkins et al., 1990; Nicoletti et al., 1990; Theibert et al., 1991, 1992), and one of them has been identified as the C2B domain of synaptotagmin (Fukada et al., 1994), a major calcium-sensor for regulating exocytosis in neurons. InsP6 also binds with high affinity to clathrin assembly proteins, the adaptor proteins (AP)-2 and AP-3 (Beek and Keen, 1991; Theibert et al., 1992; Timerman et al., 1992; Voglmaier et al., 1992), thereby inhibiting clathrin assembly (Beek and Keen, 1991; Norris et al., 1995; Ye et al., 1995). In fact, InsP6 has been proposed to participate in vesicle trafficking and

Abbreviations: AP, adaptor protein; AMPA, (RS)-α-amino-3-hydroxy-5-methylisoxazole-propionate; DPGA, 2,3-diphospho-D-glyceric acid; GluR, glutamate receptor; IGF II, insulin-like growth factor receptor II; InsPs, inositol phosphates; InsP3, D-myo-inositol 1,4,5-trisphosphate; InsP5, D-myo-inositol 1,3,4,5,6-pentakisphosphate; InsP6, D-myo-inositol 1,2,3,4,5,6-hexakisphosphate; InsS6, D-myo-inositol 1,2,3,4,5,6-hexakissulfate; LTP, long-term potentiation; NMDA, N-methyl-D-aspartate; OD, optical density; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PSD, postsynaptic density; SITS, 4-acetanido-4′-isothiocyanato-stilbene-2,2′-disulfonic acid; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone.

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receptor compartmentalization (Sasakawa et al., 1995), and the demonstration that microinjection of InsP6 into cells can inhibit vesicle trafficking is consistent with the involvement of an inositide-binding site in this process (Llinás et al., 1994). The concept was also recently strengthened by the observation that increased InsP6 in response to G protein-coupled receptor signals blocks the recruitment of AP-2 and clathrin-dependent endocytosis of Na^+,K^+-ATPase activity in renal epithelial cells (Ogimito et al., 2000).

Glutamate receptors (GluRs), which are major excitatory receptors within the central nervous system, are the object of particular attention, since their regulation appears to be crucial for controlling synaptic function. For instance, GluRs have been proposed to control fast excitatory neurotransmission at glutamatergic synapses, and their excessive activation has been demonstrated to be potently neurotoxic (Seeburg, 1993; Hollmann and Heinemann, 1994; Lipton and Rosenberg, 1994). N-methyl-D-aspartate (NMDA) and non-NMDA (kainate and α-amino-3-hydroxy-5-methylisoxazole-propionate; AMPA) receptors are two families of ionotropic receptors activated by glutamate (Dingledine et al., 1999). Among several important functions, the NMDA receptor has been implicated in the induction of long-term potentiation (LTP), an electrophysiological model of learning and memory (Bliss and Collingridge, 1993). However, alterations in AMPA receptors appear to be primarily responsible for the enhanced synaptic current observed during expression of LTP (Kauer et al., 1988; Muller and Lynch., 1988; Davies et al., 1989). In this context, studies have shown that LTP expression may involve the uncovering of functional AMPA receptors in hippocampal slices that, prior to LTP, were either not present in postsynaptic membranes or were electrophysiologically silent (Isaac et al., 1995; Liao et al., 1995). Consistently, recent reports have proposed that regulation in the surface expression of AMPA receptors at individual synapses may contribute to the changes in synaptic strength that occur during LTP (Shi et al., 1999; Hayashi et al., 2000).

Although the detailed molecular mechanisms by which AMPA receptors are regulated during synaptic plasticity remain intensely debated, it was suggested recently that the surface expression of these receptors might undergo endocytosis through a clathrin-dependent process (Man et al., 2000; Wang and Linden, 2000). It was also reported that GluR2 subunits coimmunoprecipitate with adaptor protein 2 (AP-2) (Man et al., 2000), indicating that inositol lipids could play a role in the regulation of AMPA receptors. Here, we used in vitro receptor autoradiography, subcellular fractionation, and immunoblotting to study the effect of InsP6 on both AMPA and NMDA receptors. Our results suggest that InsP6 treatment of rat brain sections selectively regulates the subcellular distribution of AMPA receptors.

MATERIALS AND METHODS

Tissue Preparation and Chemicals

Male Sprague-Dawley rats (Anilab, Inc., St. Foy, Quebec, Canada) were kept in a temperature- and humidity-controlled environment and exposed to a 12-h light/12-h dark cycle, with standard rat chow and water ad libitum. The animals were housed in a facility that met Canadian Council of Animal Care guidelines. For binding studies, 4-month-old rats were sacrificed by decapitation after methoxyflurane anesthesia. Their brains were quickly removed and frozen in isopentane (methyl butane) at −20°C, and then kept at −70°C. Frontal or horizontal 10-μm-thick sections were cut in a cryostat, thaw-mounted onto chrome-alum gelatin-coated slides, and stored at −70°C until the day of use. 2,3-diphospho-D-glyceric acid (DPGA), D-myo-inositol 1,4,5-trisphosphate (InsP3), D-myo-inositol 1,3,4,5,6-pentakisphosphate (InsP5), D-myo-inositol 1,2,3,4,5,6-hexakissulfate (InsS6), and InsP6 were all obtained from Sigma (St. Louis, MO).

Quantitative Glutamate Receptor Autoradiography

Adjacent sections were preincubated at 35°C for 60 min in 50 ml of Tris-acetate buffer (100 mM, pH 7.4) containing 100 μM EGTA with and without InsP6 (or other compounds). In some experiments, preincubation with InsP6 was also performed in the presence of heparin (2.0 U/ml). After washing in 50 ml of Tris-acetate buffer (100 mM, pH 7.4) containing 100 μM EGTA, the sections were processed for AMPA receptor binding, as described previously (Tocco et al., 1992). In brief, sections were incubated for 45 min at 0–4°C in Tris-acetate buffer (50 mM, pH 7.4; 100 μM EGTA) containing 50 mM potassium thiocyanate and 75 nM ^{3}H-AMPA (specific activity 53 Ci mmol^{-1}, NEN-Dupont, Wilmington, DE). Nonspecific binding was defined as binding measured in the presence of 1 mM quisqualate. At the end of incubation, the sections were rinsed twice in cold incubation buffer for 10 s and once for 5 s in 50% of the same buffer, followed by three dips in distilled water. They were rapidly either wiped off the slides with GF/C filters and filter radioactivity was counted in a liquid scintillation counter, or dried under a stream of warm air and used for autoradiography. Saturation kinetics were performed at various concentrations of ^{3}H-AMPA (20 nM to 6 μM) in area CA_1 of the hippocampus, and the autoradiographs were analyzed by the Inplot program from GrafPad to generate Kd and Bmax values.

For ^{3}H-glutamate binding to NMDA receptors, sections were incubated with 100 nM of ^{3}H-glutamate (51 Ci mmol^{-1}, NEN-Dupont) for 45 min at 0–4°C in Tris-acetate buffer (50 mM, pH 7.4; 50 μM EGTA) containing 5 μM AMPA, 1 μM kainic acid, and 10 μM quisqualate to eliminate glutamate binding to non-NMDA sites, and 100 μM SITS (4-acetanido-4′-isothiocyanatostilbene-2,2′-disulfonic acid) to block glutamate uptake sites. Nonspecific binding was defined as binding measured in the presence of 1 mM glutamate. The sections were rinsed twice in incubation buffer for 15 s and once for 5 s in 50% of the same buffer, followed by three dips in distilled water. They were dried as described above for AMPA binding. Dried sections as well as tritium standards (ARC, St. Louis, MO) were exposed to tritium-sensitive film (Amersham Hyperfilm) for 7 days (^{3}H-AMPA) or 14 days (^{3}H-glutamate). The films were processed in Sigma developer and

fixer. The optical densities of different brain regions were converted to radioactive units, using tritium standards after measurement with an image analysis system (Imaging Research, MCID, St. Catherines, Ontario, Canada). ANOVA was followed by Scheffe's post hoc analysis with the conventional criterion for statistical significance.

Electrophoresis and Immunoblotting

Horizontal 30-μm sections were cut in a cryostat and thaw-mounted in chrome-alum gelatin-coated slides. Adjacent sections were preincubated for 60 min in 50 ml of Tris-acetate buffer (100 mM, pH 7.4) containing 100 μM EGTA at 35°C with or without 200 μM InsP6. Tissue was collected in 0.32 M sucrose containing several protease inhibitors (leupeptin 5 μM, phenylmethylsulfonyl fluoride (PMSF) 200 μM, and N-tosyl-L-phenylalanine chloromethyl ketone (TCPK) 1 μg/ml), and homogenized with a glass-Teflon homogenizer. The homogenates were centrifuged to obtain crude mitochondrial/synaptic (P2) and microsomal (P3) membrane fractions (Henley, 1995). Briefly, homogenates were centrifuged at 1,000*g* for 10 min, and the supernatants were centrifuged at 11,500*g* for 20 min. The resulting pellet, P2, was defined as the synaptosomal fraction. The pellet P3, obtained by supernatant centrifugation at 100,000*g* for 1 h, was defined as the microsomal fraction. The P2 and P3 fractions were resuspended in Tris-acetate buffer (50 mM, pH 7.4, with the same concentrations of inhibitors as above), and protein concentrations in each fraction were measured by Bio-Rad protein assay. In some experiments, Triton X-100 extraction of crude synaptic membrane fractions was performed to isolate postsynaptic densities (PSDs). Aliquots of P2 fractions (about 100 μg proteins) were treated with 1 ml of Triton extraction buffer (1% Triton X-100, 40 mM Tris, 150 mM NaCl, 1 mM EDTA, 50 μM leupeptin, 100 μM PMSF, and 2 μg/ml aprotinin, pH 7.4) on ice for 15 min with intermittent vortexing. The extraction was centrifuged at 16,000*g* for 20 min, and the resulting pellet was defined as the Triton-insoluble fraction.

Western blot analysis was conducted on membrane fractions obtained from control and InsP6-treated sections. Aliquots of P2 and P3 fractions were subjected to SDS-PAGE (8% polyacrylamide), according to the method of Laemmli (1970). Proteins were transferred onto nitrocellulose membranes, as described previously (Towbin et al., 1979). To block nonspecific sites, the membranes were first incubated for 1 h at room temperature in phosphate-buffered saline (PBS) containing 5% dry nonfat milk. The membranes were incubated with primary antibodies against GluR1 (0.5 μg/ml) or GluR2 (0.5 μg/ml) (Upstate Biotechnology, Lake Placid, NY) in PBS containing 5% dry nonfat milk. Bands corresponding to GluR1 or 2 were detected with an alkaline phosphatase-conjugated secondary antibody (Roche Molecular Biochemicals). The optical density (OD) of the immunoreactive bands was quantified with a computer imaging system (Imaging Research). Here again, ANOVA was followed by Scheffe's post hoc analysis with the conventional criterion for statistical significance.

RESULTS

InsP6-Induced Changes in GluR Binding in Rat Brain Sections

We first assessed the effects of incubating rat brain sections with increasing InsP6 concentrations on the binding properties of AMPA receptors. Horizontal sections were preincubated in Tris-acetate buffer in the presence of various InsP6 concentrations (1–300 μM), and ^{3}H-AMPA binding was then measured by wiping off the sections to evaluate the amount of specifically bound radioactivity. InsP6 produced a dose-dependent increase of specific ^{3}H-AMPA binding in these rat brain sections, with a maximal elevation of about 50% above control values at 100 μM InsP6 (solid circles, Fig. 1A). Its effect was temperature-dependent, as it was markedly reduced at room temperature (22°C; open circles, Fig. 1A). At a concentration of 200 μM, InsP6 caused a 50 ± 4% increase of specific ^{3}H-AMPA binding in sections preincubated at 35°C, while it had no action in sections preincubated at room temperature (4 ± 4%; n = 6, $P < 0.05$, Student's *t*-test).

Brain sections were next preincubated with 200 μM InsP6 and subjected to quantitative ^{3}H-AMPA ligand binding autoradiography. Visual examination of autoradiography clearly showed a marked increase of ^{3}H-AMPA binding in the hippocampus, cerebellum, and other brain structures after InsP6 treatment (Fig. 1B). The rise in ^{3}H-AMPA binding elicited by InsP6 preincubation was distributed uniformly in the various regions of the hippocampus (Fig. 1C). InsP6 produced a 20–30% augmentation of ^{3}H-AMPA binding in CA_1 and CA_3 strata radiatum and oriens as well as in the dentate gyrus. It also elicited a moderate increase of about 15% in ^{3}H-AMPA binding in the internal cortex and, among all the regions analyzed, the cerebellum showed the highest elevation (88%; see Fig. 1D). Saturation experiments at equilibrium were performed to determine whether the increase in ^{3}H-AMPA binding elicited by InsP6 was the result of alterations in receptor affinity or maximal number. The saturation kinetics of binding in the strata radiatum of the CA_1 region yielded a linear Scatchard plot, suggesting the existence of a single binding site (Fig. 2). In this region, the InsP6-induced increase in ^{3}H-AMPA binding was not due to a change in affinity of AMPA receptors (Kd = 145 ± 26 nM in control vs. Kd = 158 ± 14 nM in InsP6-treated sections; means ± SEM of six experiments), but to a rise in the maximal number of sites (Bmax = 7.5 ± 0.34 in control vs. 11.6* ± 0.3 pmol/mg of protein in InsP6-treated sections; means ± SEM of six experiments, $P < 0.01$, Student's *t*-test). It should be mentioned that, in the present study, we failed to detect any high-affinity sites (Fig. 2). This was probably due to the preincubation of sections at 35°C; high-affinity AMPA-binding sites have been shown to represent cytoplasmic receptors, and to be easily washed out by preincubation of tissue sections at 35°C (Standley et al., 1998).

Ligand-binding autoradiography with ^{3}H-glutamate was also used to test the effects of InsP6 on the binding properties of NMDA receptors in various brain regions (Fig. 3A). As illustrated in Figure 3B,C, quantification and averaging of the data obtained from several sections indicated that no significant changes in ^{3}H-

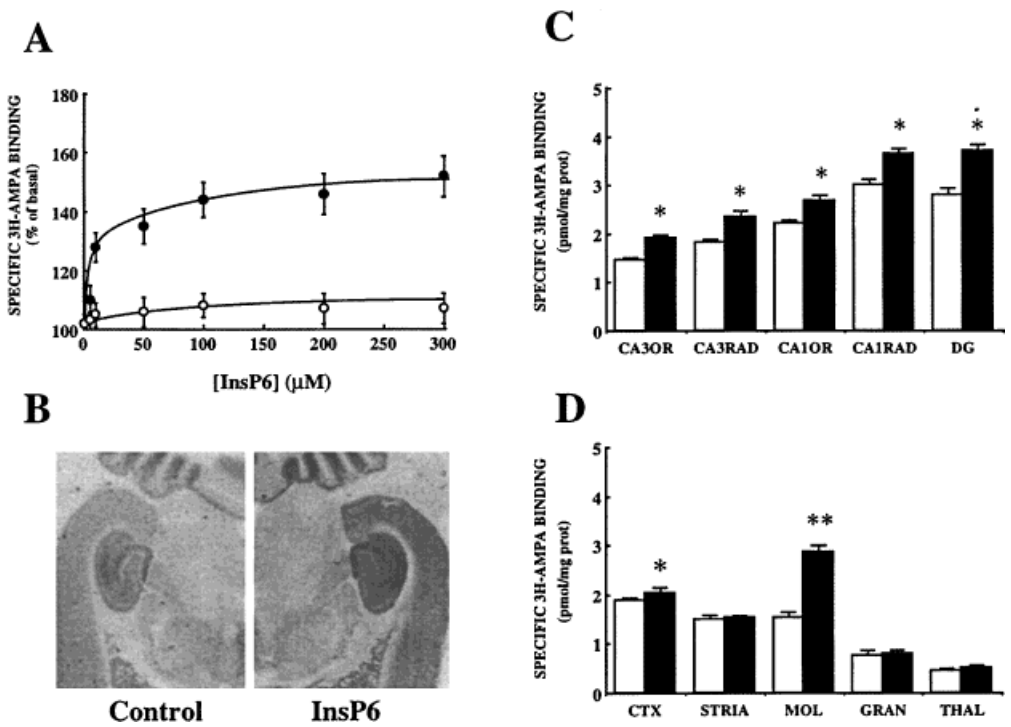

FIGURE 1. Modulation of AMPA receptor binding by InsP6. A: Adjacent horizontal sections at the level of the dorsal hippocampus were preincubated at 35°C (solid circles) or 22°C (open circles) for 1 h with increasing concentrations (1–300 μM) of InsP6. The results represent InsP6-induced modulation of ^{3}H-AMPA binding and are expressed as percent of basal binding determined in sections preincubated with buffer alone. The data are means ± SEM of values obtained from at least 5 rats with three different measurements per rat. B: Computer-generated images of autoradiograms of ^{3}H-AMPA binding in rat brain sections preincubated at 35°C for 1 h in the absence or presence of 200 μM InsP6. C: Autoradiographs like those shown in B were analyzed with an image analysis system for the various hippocampal regions (OR, stratum oriens; RAD, stratum radiatum; DG, molecular layer of the dentate gyrus). The results obtained for the different regions of control (open bars) and InsP6-treated (200 μM; solid bars) sections are expressed in pmol/mg of protein. D: Same as C, but representing ^{3}H-AMPA binding to non-hippocampal structures (CTX, inner layers III–VI of the parieto-temporal cortex; STRIA, striatum; MOL, molecular layer of the cerebellum; GRAN, granular layer of the cerebellum; THAL, thalamic nuclei). The data are means ± SEM of values obtained from at least 6 rats with at least eight different measurements per rat for each of the cerebral regions examined. *$P < 0.05$, **$P < 0.01$ (Scheffe's test). InsP6-treated vs. control sections.

glutamate binding were produced by InsP6 treatment in the various brain regions analyzed. Of course, these results suggest that changes in AMPA receptors are not the result of a chemical artifact of tissue preparation.

Subcellular Localization of GluR Subunits After InsP6 Treatment of Rat Brain Sections

Frozen-thawed brain sections (30 μm thick) were incubated at 35°C in the presence and absence of 200 μM InsP6 for 60 min. After preincubation, they were homogenized and subjected to subcellular fractionation. Samples from crude mitochondrial/synaptic (P2) and microsomal (P3) fractions were immunoblotted with antibodies against the C-terminal domains of GluR1 or GluR2, as reported previously (Bi et al., 1997, 1998). Figure 4A shows that incubation of rat brain sections with InsP6 resulted in an increase of 105-kDa species of GluR1 and GluR2 subunits in the mitochondrial/synaptic (P2) fraction. The mean (± SEM) OD values for both GluR1 (0.64 ± 0.05 in control vs. 0.84 ± 0.06 in InsP6-treated sections; n = 8, $P < 0.01$) and GluR2 (0.47 ± 0.05 in control vs. 0.61 ± 0.05 in InsP6-treated sections; n = 8, $P < 0.01$) subunits were elevated by about 30% after InsP6 treatment of rat brain sections. However, while the levels of these two subunits were enhanced in the P2 fraction, they were reduced (also by about 30%) in the microsomal (P3) fraction (Fig. 4A). It should be mentioned that, in homogenates containing both P2 and P3 fractions, we failed to detect any changes in GluR1 subunits after InsP6 treatment (0.52 ± 0.06 in control vs. 0.55 ± 0.04 in InsP6-treated sections; n = 4), reinforcing the notion that apparent increases in GluR subunits observed in P2 fractions are due to corresponding reductions in P3 fractions. Another set of experiments was conducted using anti-NR1 and anti-NR2A antibodies to test whether NMDA receptors were affected by InsP6 treatment of rat brain sections. Consistent with the binding experiments, no significant differences in the intensity of NR1 or NR2A staining were detected in mitochondrial/synaptic as well as microsomal fractions prepared from control and InsP6-treated sections (Fig. 4B).

The cellular mechanisms for the regulation of AMPA receptor properties by InsP6 remain to be understood. InsP6 is known to interact with adaptor proteins in the brain, and InsP6 binding has been demonstrated to be inhibited by heparin

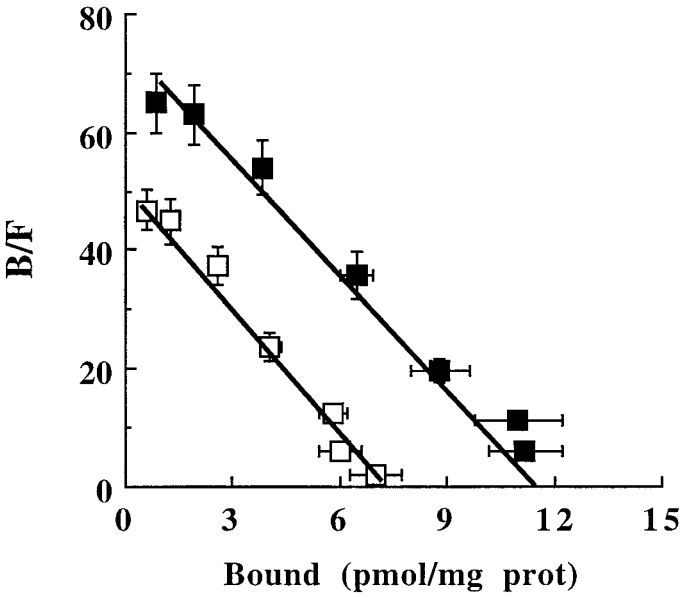

FIGURE 2. Effect of InsP6 on binding properties of AMPA receptors in the hippocampus formation. Scatchard plots of ^{3}H-AMPA binding in the stratum radiatum of the CA1 region of sections preincubated without (open squares) and with (solid squares) 200 μM InsP6. ^{3}H-AMPA binding was determined using concentrations from 20 nM to 6 μM, and autoradiography was performed as described in Materials and Methods. The data are presented as Scatchard plots (B/F, bound/free) and are analyzed by the Inplot program from GrafPad to generate Kd and Bmax; Scatchard plots are the results of experiments performed in 5 different animals.

(Theibert et al., 1991). Therefore, we examined the effect of heparin on InsP6-induced modulation of AMPA receptor properties. Preincubation of horizontal sections with heparin (2 U/ml) significantly reduced the InsP6-mediated increase of ^{3}H-AMPA binding (Fig. 5A). In addition, heparin prevented the InsP6-induced elevation of GluR1 subunits of AMPA receptors in synaptic membranes (Fig. 5B). The effect of InsP6 on GluR1 subunits was also abolished by pretreatment of rat sections with a hypertonic medium (350 mM glucose) and was not reproduced by a similar concentration of InsS6 that binds with low affinity to clathrin adaptor protein, suggesting that it requires clathrin-dependent processes (see Fig. 6A).

We also examined the effects of InsP6 treatment of rat brain sections on Triton X-100 solubility of GluR1 subunits in crude synaptic membrane (P2) preparations. In agreement with previous reports (Wenthold et al., 1996; Lu et al., 1999), levels of Triton X-100-insoluble GluR1 subunits represented about 20% of those occurring in P2 fractions (Fig. 5C). Triton-insoluble AMPA receptors possibly represent receptors incorporated in PSDs, as the pattern of proteins observed with Coomassie blue staining of Triton-insoluble fractions was very similar to that reported by others using standard PSD preparation procedures, with clear identification of spectrin, actin, and CaMKII (data not shown). InsP6-induced elevation of GluR1 subunit levels was also observed in Triton-insoluble fractions (Fig. 5C), suggesting that InsP6 plays a role in regulating AMPA receptors at PSDs.

Effects of Other Inositol Phosphates on AMPA Receptors

It might be argued that InsPs produced by InsP6 dephosphorylation during the preincubation period can account for the observed modulation of AMPA receptors by InsP6. Therefore, we examined the effect of several InsPs (InsP5, InsP3, and DPGA) on GluR1 subunit levels in P2 fractions. As shown in Figure 6, Western blotting experiments indicated a dose-dependent increase of GluR1 immunoreactivity in P2 fractions, with a maximal augmentation to about 40% of control values at 200 μM InsP6. This effect of InsP6 on GluR1 appeared to be relatively specific, since it was not reproduced by InsS6 and other phosphate compounds such as InsP5, InsP3, and DPGA. In fact, our immunoblot experiments revealed that both InsP5 and InsP3 are capable of reducing GluR1

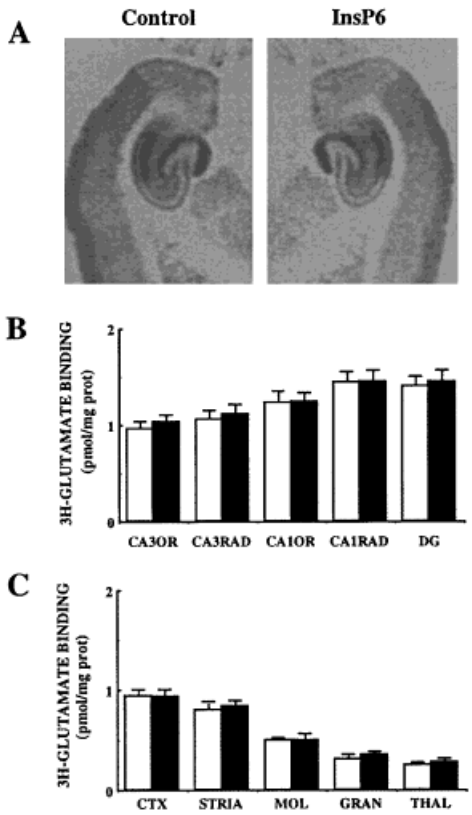

FIGURE 3. Effect of InsP6 on NMDA receptor binding. A: Computer-generated images of autoradiograms of ^{3}H-glutamate binding to NMDA receptor sites in rat brain sections preincubated at 35°C for 1 h in the absence or presence of 200 μM InsP6. B: Autoradiographs like those shown in A were analyzed with an image analysis system for the various hippocampal regions (OR, stratum oriens; RAD, stratum radiatum; DG, molecular layer of dentate gyrus). The results obtained for the different regions of control (open bars) and InsP6-treated (200 μM; solid bars) sections are expressed in pmol/mg of protein. C: Same as B but represent ^{3}H-glutamate binding to nonhippocampal structures (CTX, inner layers III–VI of the parieto-temporal cortex; STRIA, striatum; MOL, molecular layer of the cerebellum; GRAN, granular layer of the cerebellum; THAL, thalamic nuclei). The data are means ± SEM of values obtained from at least 6 rats with at least eight different measurements per rat for each of the cerebral regions examined.

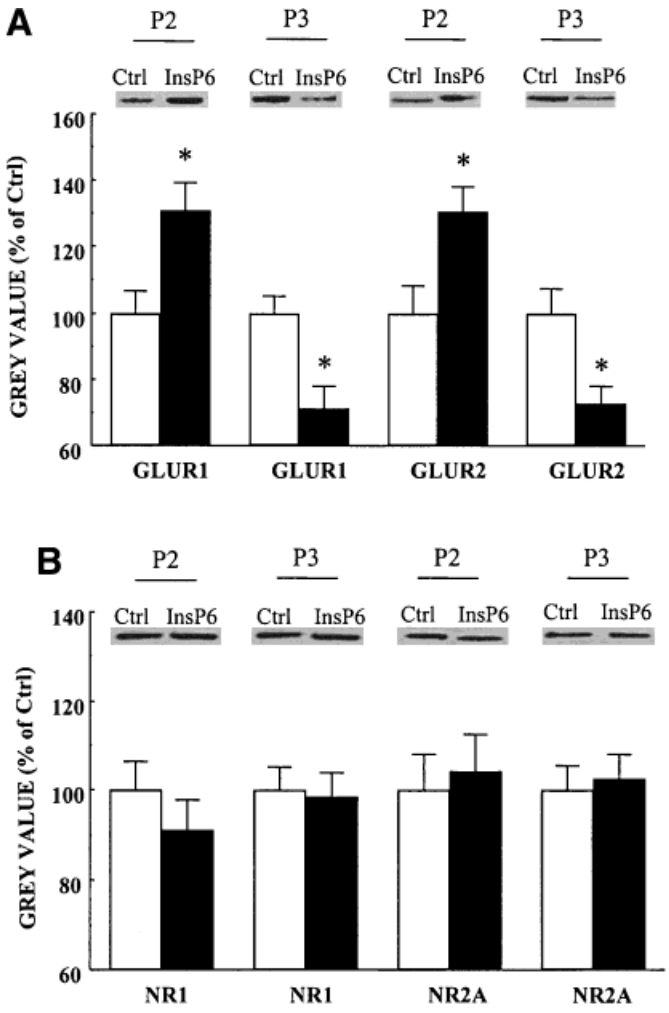

FIGURE 4. Effect of InsP6 treatment on subcellular localization of glutamate receptor subunits. A: Adjacent horizontal sections at the level of the dorsal hippocampus were preincubated at 35°C for 1 h in the absence (open bars) or presence (solid bars) of 200 μM InsP6. After incubation, mitochondrial/synaptic (P2) and microsomal (P3) fractions were prepared by subcellular fractionation. Western blots were performed using antibodies against the C-terminal domain of GluR1 and GluR2 subunits of AMPA receptors. Blots were digitized, and the intensity of the 105-kDa bands was quantified (grey values). Representative Western blots showing InsP6-induced modulation of the levels of AMPA receptor subunits in both P2 and P3 fractions are presented above the graph. B: Same as A for NR1 and NR2A subunits of NMDA receptors. Results are expressed as percent of control values and represent the means ± SEM of 6–8 experiments. *$P < 0.05$, compared with control values (Scheffe's test).

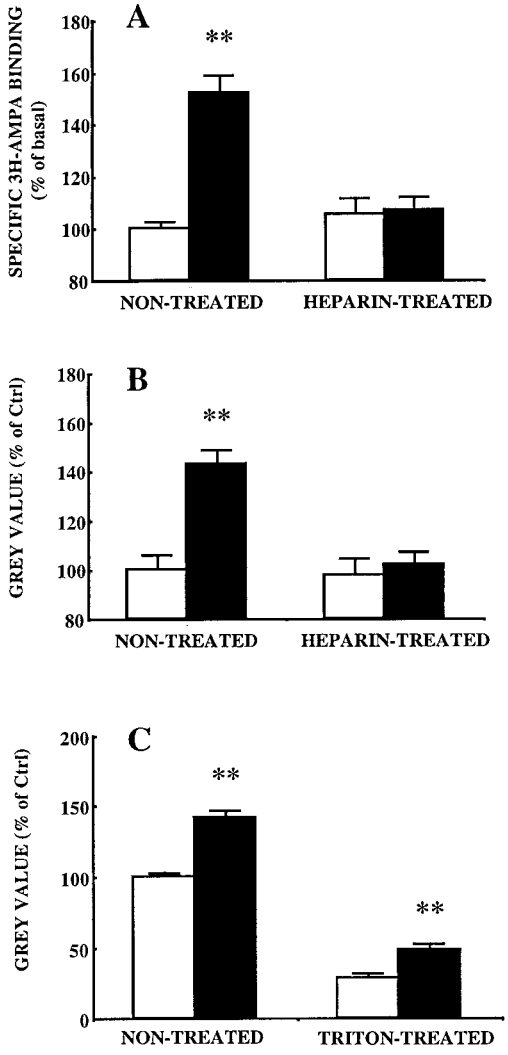

FIGURE 5. Effect of heparin and Triton X-100 on InsP6-induced modulation of AMPA receptors. A: Adjacent horizontal sections at the level of the dorsal hippocampus were preincubated at 35°C for 1 h with 2 U/ml heparin in the absence (open bars) or presence (solid bars) of 200 μM InsP6. ^{3}H-AMPA binding to whole sections was determined, as described in Materials and Methods. Results are expressed as percent of basal binding in control sections. Data are means ± SEM of values obtained from 5 rats. B: Same as A for the GluR1 subunits of AMPA receptors. Mitochondrial/synaptic (P2) fractions of rat brain sections were prepared by subcellular fractionation, and Western blots were performed using antibodies against the C-terminal domain of GluR1 subunits. Results are expressed as percent of control values and represent means ± SEM of six experiments. C: Adjacent horizontal sections at the level of the dorsal hippocampus were preincubated at 35°C for 1 h in the absence (open bars) or presence (solid bars) of 200 μM InsP6. After incubation, mitochondrial/synaptic (P2) fractions were prepared by subcellular fractionation and subjected to Triton X-100 extraction. Western blots were performed using antibodies against the C-terminal domain of GluR1 subunits. Results are expressed as percent of control values found in P2 control fractions and represent the means ± SEM of five experiments. **$P < 0.01$, compared with control values (Scheffe's test).

immunoreactivity in P2 fractions (Fig. 6), suggesting that AMPA receptors can be differentially regulated by InsPs.

DISCUSSION

In this study, we determined the effects of InsP6 treatment of frozen-thawed rat brain sections on AMPA and NMDA receptors.

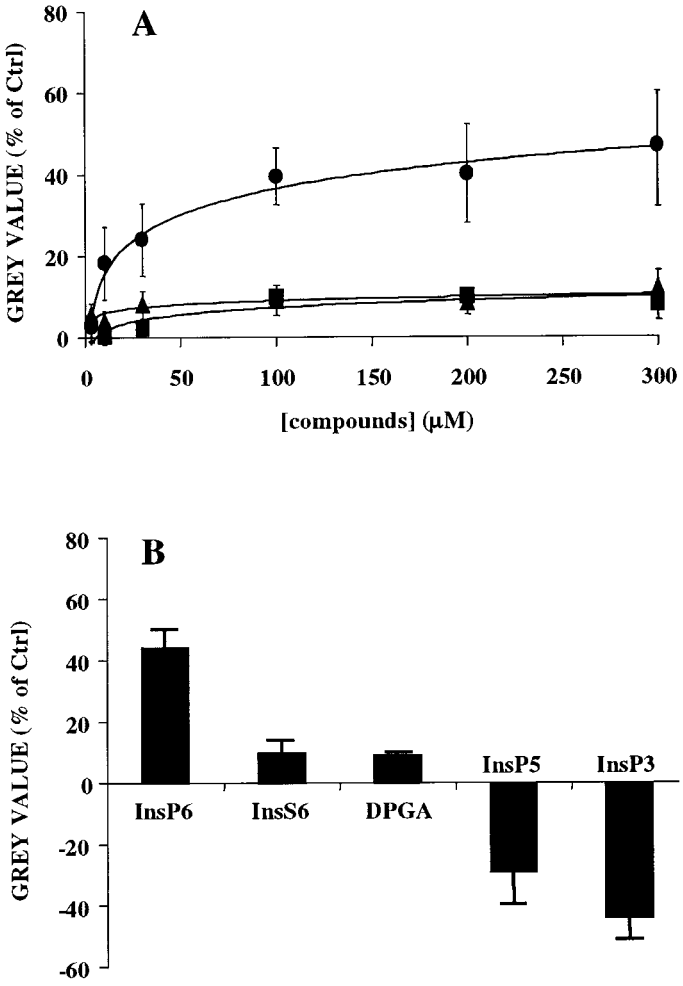

FIGURE 6. Effects of various phosphate compounds and InsS6 on GluR1 subunits. A: Adjacent horizontal sections at the level of the dorsal hippocampus were preincubated at 35°C for 1 h in the absence or presence of increasing concentrations (3–300 μM) of InsP6 (solid circles), InsS6 (closed squares), and DPGA (solid triangles). B: Same as A for sections incubated with 100 μM of various phosphate compounds and InsS6. Mitochondrial/synaptic (P2) fractions of rat brain sections were prepared by subcellular fractionation, and Western blots were performed using antibodies against the C-terminal domain of GluR1 subunits. Results are expressed as percent of control values found in P2 control fractions and represent the means ± SEM of five experiments.

Western blot and ligand-binding analysis revealed that InsP6 treatment of brain sections selectively modulated AMPA receptor properties. In particular, InsP6 produced an increase in the levels of GluR1 and GluR2 subunits of AMPA receptors in crude mitochondrial/synaptic (P2) fractions prepared from rat brain sections. In contrast, the amounts of these subunits were reduced in microsomal (P3) fractions, suggesting that AMPA receptor distribution between intracellular and plasma membrane compartments was altered by InsP6 treatment. Quantitative autoradiography of ^{3}H-AMPA binding similarly revealed that InsP6 treatment enhanced ^{3}H-AMPA binding sites in several regions, although modulation of AMPA binding appeared to be different in various brain structures. Whether regional differences in the effects of InsP6 are due to heterogeneity of AMPA receptors or to regional variations in the ability of tissues to generate the InsP6 effect is not clear. Nevertheless, the observation that AMPA receptor modulation by InsP6 was maximal in the hippocampus and cerebellum is entirely consistent with the distribution of InsP6 binding sites in these brain regions (Hawkins et al., 1990; Parent and Quirion, 1994), raising the possibility that endogenous InsP6 plays an important role in the regulation of AMPA receptors.

There are relatively few studies on the regulation of surface receptors by InsP6. With regard to the present investigation, Kar et al. (1994) reported an interaction between InsP6 and insulin-like growth factor receptor II (IGF II) in rat brain sections. InsP6 also reduced light-induced desensitization of rhodopsin (Palczewski et al., 1992), presumably through direct binding to arrestin (Palczewski et al., 1991), suggesting that InsP6 participates in the control of G-protein-coupled receptors. In agreement with this prediction, agonist-induced desensitization of substance P receptors was blocked by InsP6 in *Xenopus* oocytes (Sasakawa et al., 1994). AMPA receptors, originally identified as classical ionotropic receptors, were recently found to exhibit metabotropic activity. In particular, AMPA-receptor stimulation reduced both pertussis toxin-induced ADP ribolysation of Gαi1 and forskolin-induced cAMP elevation in cultured cortical neurons and membrane preparations (Wang et al., 1997). Interestingly, Gαi1 coimmunoprecipitated with GluR1 in AMPA-treated cultures but not in control cultures, suggesting the involvement of ionotropic AMPA receptors in metabotropic signaling cascades. The mechanism of interaction between AMPA receptors and G proteins is still unclear, although an AP might be involved. One indication that this idea might be true is the recent observation that GluR2 subunits coimmunoprecipitated with AP (Man et al., 2000).

The recognized interaction of InsP6 with AP-2 calls attention to the possibility that InsP6 might contribute to the regulation of AMPA receptor levels in synaptic membranes. It is well-recognized that AP-2 binds to the cytoplasmic tail of a number of membrane receptors and is involved in the internalization of neurotransmitter receptors. In particular, AP-2 is known to interact with receptors for IGF II and to mediate the endocytosis of IGF II as well as other signaling peptides, such as transforming growth factor β (Glickman et al., 1989; Kar et al., 1994). Once recruited to the inner surface of the plasma membrane, AP-2 is likely to initiate the formation of clathrin-coated pits by triggering the assembly of clathrin triskelion subunits into a polygonal lattice that causes bending of the membrane into the coated pit structure (Heuser and Keen, 1988; Mahaffey et al., 1990). Clathrin-coated pits detach from the plasma membrane by a GTP-dependent fission reaction mediated by the GTPase dynamin, and the resulting coated membrane vesicles become internalized (Koenig and Ikeda, 1989). InsP6, by interacting with AP-2, inhibits clathrin assembly and vesicle trafficking, suggesting its influence on receptor compartmentalization (Sasakawa et al., 1995). In the present investigation, the levels of some GluR subunits were increased in crude mitochondrial/synaptic (P2) fractions and decreased in microsomal (P3) fractions following InsP6 treatment. As predicted, heparin was found to block the InsP6-induced change of AMPA receptor compartmentalization, suggesting that InsP6 modulation of

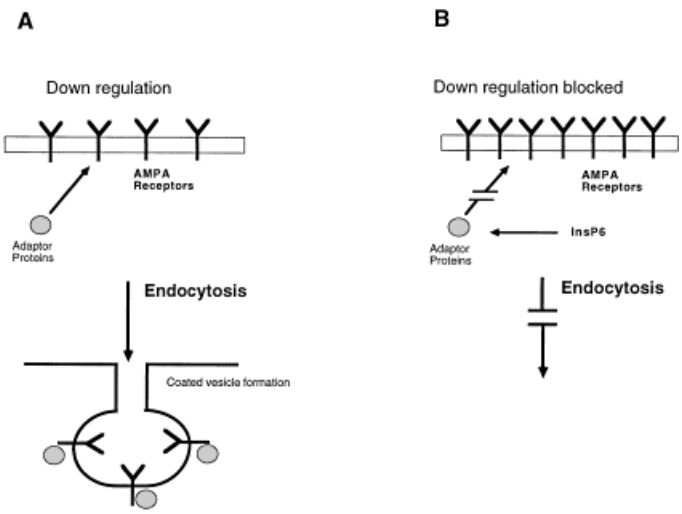

FIGURE 7. Working model of InsP6-mediated modulation of AMPA receptor properties. A: Downregulation of AMPA receptors is initiated by internalization, leading to a loss of receptors in plasma membranes. One of the components of receptor internalization is the clathrin assembly protein AP-2, which is required for coated pits and coated vesicle formation. B: InsP6 binding to AP-2 would block clathrin assembly and receptor internalization, promoting the maintenance of AMPA receptors in synaptic plasma membranes.

AMPA receptors is due to an interaction with APs. The present results, together with recent works revealing that intracellular AMPA receptors are colocalized with EPS15 and AP-2 (two components of clathrin-coated pits), support the contention that AMPA receptors might undergo internalization into clathrin-coated vesicles (Carroll et al., 1999; Man et al., 2000; Wang and Linden, 2000). A putative biochemical model that accounts for the control of AMPA receptors by InsP6 is represented in Figure 7. It should be noted that this model assumes that internalization of AMPA receptors takes place in rat brain sections in the absence of an exogenous source of energy. Further studies are needed to verify this assumption.

Our results clearly indicate that the changes produced by InsP6 on the AMPA subtype of glutamate receptors are not due to chemical artifact, as InsP6 treatment did not modify either ^{3}H-glutamate binding to NMDA receptors or the levels of NR1 and NR2A subunits in either P2 or P3 fractions. These data are in good agreement with previous studies showing that, while NMDA receptors are not modified by various biochemical and physiological manipulations, AMPA receptors are easily altered under such conditions. For instance, pretreatment of telencephalic membranes with certain calcium-dependent phospholipases selectively enhanced agonist binding to AMPA but not NMDA receptors (Massicotte and Baudry, 1990; Massicotte et al., 1990). The reasons for such differences in behavior of AMPA and NMDA receptors after InsP6 treatment are presently unknown. Different subunit compositions and/or an association of receptors with synaptic or cytoskeletal proteins might explain the selective action of InsP6. In this regard, it is noteworthy that intracellular proteins, such as glutamate receptor interacting protein and N-ethylmaleimide-sensitive fusion protein, were recently identified as AMPA receptor-interacting proteins (Lin and Sheng, 1998; Nishimune et al., 1998). We need to determine whether InsP6 treatment has any effect on the interactions of AMPA receptor subunits with any of these proteins.

The ability of InsP6 to modulate AMPA receptor properties might greatly facilitate elucidation of the molecular mechanisms responsible for activity-dependent regulation of AMPA receptor-targeting synapses and synaptic membranes. It is now established that experimental manipulations of neuronal activity can cause redistribution of AMPA receptors toward or away from synaptic sites (Lissin et al., 1998; Carrol et al., 1999). The present biochemical observations are consistent with the notion of a rapid cycling of AMPA receptors in and out of postsynaptic membranes (Luscher et al., 1999; Shi et al., 1999) and with the demonstration that these receptors might be regulated by dynamin-dependent endocytosis (Carroll et al., 1999). On the other hand, a role for InsP6 in regulating AMPA-receptor internalization is consistent with studies on synaptic plasticity that have indicated that membrane fusion events (Lledo et al., 1998), insertion of functional AMPA receptors (Isaac et al., 1995; Liao et al., 1995), and ^{3}H-AMPA binding (Maren et al., 1993) are modulated during LTP. The fact that InsP6 levels can be rapidly modified in cultured cerebellar granule cells upon depolarization with high potassium (Sasakawa et al., 1993) strongly suggests that neuronal activity and InsP6-induced modulation of AMPA receptor number at postsynaptic membranes might be interrelated. This is an intriguing possibility that warrants further investigation.

Our results underscore that AMPA-receptor distribution in neurons might be under the control of InsP6 production. Of course, the functional significance of this modulation remains unknown; further experiments on the interactions of AMPA receptors with InsP6 should provide interesting information on the control of synaptic operation. In particular, additional experiments remain to be performed to establish whether the observed action of InsP6 can also be exerted during ligand-mediated endocytosis of AMPA receptors in neurons. Of course, future work will also include elucidation of the mechanisms and functional significance underlying changes in AMPA receptor properties generated by other InsPs, such as InsP5 and InsP3.

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