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ErbB1 RECEPTOR LIGANDS ATTENUATE THE EXPRESSION OF SYNAPTIC SCAFFOLDING PROTEINS, GRIP1 AND SAP97, IN DEVELOPING NEOCORTEX

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Abstract—Scaffolding proteins containing postsynaptic density-95/discs large/zone occludens-1 (PDZ) domains interact with synaptic receptors and cytoskeletal components and are therefore implicated in synaptic development and plasticity. Little is known, however, about what regulates the expression of PDZ proteins and how the levels of these proteins influence synaptic development. Here, we show that ligands for epidermal growth factor receptors (ErbB1) decrease a particular set of PDZ proteins and negatively influence synaptic formation or maturation. In short-term neocortical cultures, concentrations of epidermal growth factor and amphiregulin (2–9 pM) decreased the expression of glutamate receptor interacting protein 1 (GRIP1) and synapse-associated protein 97 kDa (SAP97) without affecting postsynaptic density-95 (PSD-95) levels and glial proliferation. In long-term cultures, epidermal growth factor treatment resulted in a decrease in the frequency of pan-PDZ-immunoreactive aggregates on dendritic processes. A similar activity on the same PDZ proteins was observed in the developing neocortex following epidermal growth factor administration to rat neonates. Immunoblotting revealed that administered epidermal growth factor from the periphery activated brain ErbB1 receptors and decreased GRIP1 and SAP97 protein levels in the neocortex. Laser-confocal imaging indicated that epidermal growth factor administration suppressed the formation of pan-PDZ-immunoreactive puncta and dispersed those structures *in vivo* as well. These findings revealed a novel negative activity of ErbB1 receptor ligands that attenuates the expression of the PDZ proteins and inhibits postsynaptic maturation in developing neocortex.

Key words: EGFR, HER1, amphiregulin, synaptic elimination, cerebral cortex, postsynaptic, schizophrenia.

Proteins carrying PDZ (postsynaptic density-95/discs large/zone occludens-1) domains are referred to as PDZ proteins, which have a modular organization and often function as scaffolding proteins. Recent studies have indicated that these proteins interact with various types of synaptic proteins, such as ion channels, signal transducers, and cytoskeletal components, at the postsynaptic density (PSD), and regulate neural transmission (Kim and Sheng, 2004). The family of PDZ proteins includes PSD-95, PSD-93/chapsyn-110, synapse-associated protein 97 kDa (SAP97), SAP102, glutamate receptor interacting protein (GRIP), and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) binding protein (ABP/GRIP2) (Nagano et al., 1998). A number of studies have shown that PSD-95 binds directly to *N*-methyl-D-aspartate receptors (NMDARs) and Shaker-type K^+ channels, thereby playing an important role in the functional localization of these proteins by linking the receptors to the cytoskeleton (Valtschanoff and Weinberg, 2001; Lei et al., 2001; Petersen et al., 2003; Lin et al., 2004). SAP97 binds to AMPARs and similarly modulates their subcellular dynamics (Leonard et al., 1998; Valtschanoff et al., 2000; Wu et al., 2002; Ko et al., 2003). Thus, PDZ proteins perform key roles in synaptic function and plasticity. Little is known, however, about the molecular regulators of PDZ protein expression during synaptic development. Growth factors and neurotrophins, such as brain-derived neurotrophic factor (BDNF) and neuregulin-1 (NRG1), influence synaptic formation and maturation in part by regulating the expression of glutamate and acetylcholine receptors and controlling their subcellular localizations (Ozaki et al., 1997; Narisawa-Saito et al., 1999a, 2002; Cotrufo et al., 2003). We previously reported that endogenous and exogenous BDNF enhances AMPAR function by increasing PDZ protein levels and their interactions with AMPARs in developing neocortical neurons (Jourdi et al., 2003). Cotrufo et al. (2003) reported that nerve growth factor up-regulates the expression of PSD-95 and GRIP in developing neocortex.

In contrast to BDNF, we observed that epidermal growth factor (EGF) suppresses AMPAR expression in cultured neocortical neurons, implying that EGF negatively affects synaptic development and function (Narisawa-Saito et al., 1999a). In the present study, we analyzed the negative role of ErbB1 receptor ligands in the regulation of the expression of PDZ proteins. EGF binds to an ErbB1

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Abbreviations: AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor; ARIA, acetylcholine receptor-inducing activity; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; DIV, days *in vitro*; EGF, epidermal growth factor; GFAP, glial fibrillary acidic protein; GRIP, glutamate receptor interacting protein; NMDAR, *N*-methyl-D-aspartate receptor; NRG1, neuregulin-1; NSE, neuron specific enolase; PDZ, postsynaptic density-95/discs large/zone occludens-1; PSD, postsynaptic density; RT-PCR, reverse transcriptase–polymerase chain reaction; SAP97, synapse-associated protein 97 kDa; T-TBS, Tween 20 in Tris-buffered saline.

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receptor tyrosine kinase that belongs to the vertebrate ErbB family. This receptor also interacts with a variety of receptor ligands including transforming growth factor α, amphiregulin, heparin-binding EGF-like growth factor, betacellulin, epiregulin and epigen (Harris et al., 2003). These ErbB1 receptor ligands regulate the proliferation of astrocytes and neuronal progenitor cells in the CNS (Yamada et al., 1997). In addition, ErbB1 receptor ligands have a trophic effect on midbrain dopaminergic neurons (Ferrari et al., 1991; Casper et al., 1994; Iwakura et al., 2005). Although ErbB1 receptors are known to be distributed in a variety of postmitotic neurons (Werner et al., 1988), the biological role of ErbB1 receptor ligands in the brain remains to be characterized. In the present study, we investigated the attenuation of the expression of PDZ proteins by ErbB1 receptor ligands both in neuron-enriched cultures and *in vivo*. The potential contribution of this activity to synaptic formation or maturation in the developing neocortex is discussed.

EXPERIMENTAL PROCEDURES

Animals

Sprague–Dawley rats were purchased with dams from SLC Ltd. (Shizuoka, Japan), and maintained under a 12-h light/dark cycle with free access to food and water. All of the experiments described were performed in accordance with the local and international guidelines on the ethical use of laboratory animals. Efforts were made to minimize the number of animals and their suffering.

Cell culture

The neocortices of day-19 rat embryos were dissected. The tissues were digested with papain (1 mg/ml), and mechanically dissociated with the aid of a plastic pipette. The dissociated cells were plated onto poly-D-lysine-coated dishes at relatively low cell densities (100–200 cells/mm^2). Neocortical neurons were grown in serum-free Dulbecco's modified Eagle medium containing nutrient mixture N2 (Narisawa-Saito et al., 1999a). This procedure reduced astroglial contamination to less than 5% of the total cell population until 7 days *in vitro* (DIV) (Narisawa-Saito et al., 1999a). For biochemical assay, cultures were supplemented daily with purified recombinant human EGF (20 pg/ml) or amphiregulin (200 pg/ml) for 6 days. An ErbB1 receptor inhibitor, PD153035 (100 nM, Tocris Cookson Inc., St. Louis, MO, USA) was added 2 h before each EGF treatment. Alternatively, EGF treatment was extended until DIV12 for immunocytochemistry (see below).

Immunoblotting

Tissues or cells were lysed in SDS lysis buffer containing 1% SDS, 20 mM Tris–HCl (pH 7.4), 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 0.5 mM phenylarsine oxide, and 1 mM phenylmethylsulfonyl fluoride. The lysates were boiled for 3 min, and then clarified by centrifugation. The protein concentrations of the supernatants were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein were then subjected to electrophoresis on 7.5% or 10% SDS–polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA) in 0.1 M Tris base, 0.192 M glycine and 20% methanol using a semi-dry electrophoretic transfer system. The membranes were blocked overnight at 4 °C with 0.1% Tween 20 in Tris-buffered saline (T-TBS) containing 5% bovine serum albumin. Membranes were then probed with the following primary antibodies: anti-SAP97 (1:800 dilution, StressGen, Victoria, BC, Canada), anti-GRIP1 (1:1000, BD Transduction Laboratory, San Diego, CA, USA), anti-pan-PDZ (1:1000, Upstate Biotechnology, Waltham, MA, USA), anti-PSD-95 (1:2000, Upstate Biotechnology), anti-β-actin (1:1000, Chemicon Int., Temecula, CA, USA), anti-neuron specific enolase (NSE; 1:1000, Polysciences, Inc., Warrington, PA, USA), anti-glial fibrillary acidic protein (GFAP; 1:4000, DakoCytomation, Glostrup, Denmark),

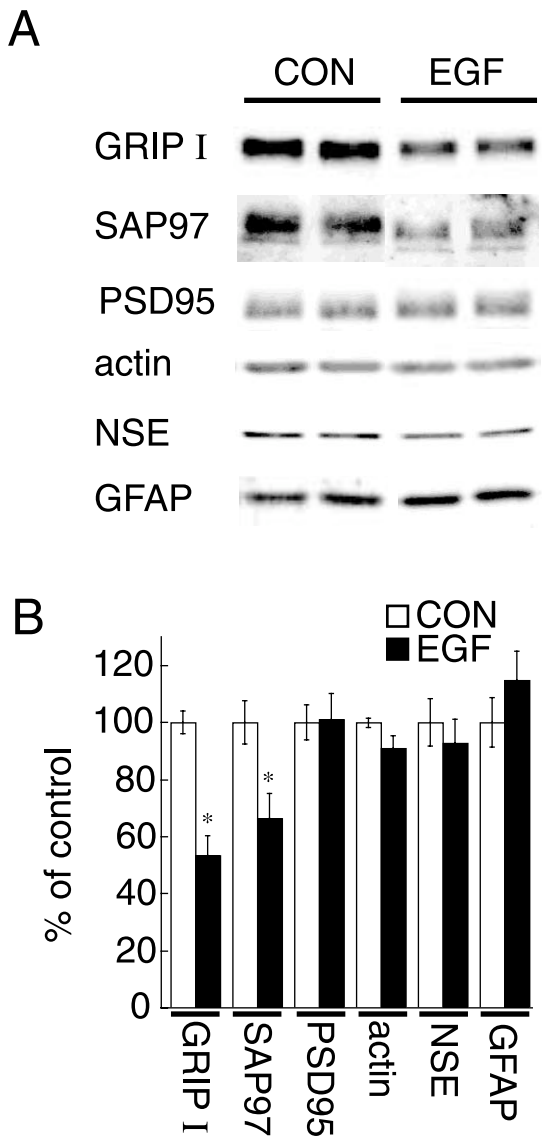

Fig. 1. Effects of EGF on the expression of GRIP1 and SAP97 proteins in neocortical cultures. Neocortical cultures were prepared from day-19 rat embryos and maintained in the presence or absence of EGF for 7 days (20 pg/ml, $n=5$ sister cultures for each condition). (A) Protein was extracted from sister cultures and subjected to immunoblotting with antibodies specific for GRIP1, SAP97, PSD-95, β-actin, NSE, and GFAP. Typical immunoblots are shown for display. (B) The intensity of immunoreactivity was quantified by densitometry. Data represent the mean±S.E.M. ($n=5$). * $P<0.05$ vs. control (Student's *t* test). CON, control. Note that there were no significant differences in MAP2-positive cell densities at 7 DIV (108±10 cells/mm^2 for control cultures and 100±10 cells/mm^2 for EGF-treated cultures, $n=4$) or in protein yields (176±14 μg/dish for control cultures and 189±4 μg/dish for EGF-treated cultures, $n=5$). The densitometric analysis was re-evaluated by independent immunoblotting with serial dilution of samples (data not shown).