Neuroscience Letters 504 (2011) 170–175

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Clavulanic acid increases dopamine release in neuronal cells through a mechanism involving enhanced vesicle trafficking

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ARTICLE INFO

Article history:
Received 21 April 2011
Received in revised form 30 August 2011
Accepted 15 September 2011

Keywords:
Clavulanic acid
Dopamine
Rab4/Munc18-1
Neurotransmitter release
Depression

ABSTRACT

Clavulanic acid is a CNS-modulating compound with exceptional blood–brain barrier permeability and safety profile. Clavulanic acid has been proposed to have anti-depressant activity and is currently entering Phase IIb clinical trials for the treatment of Major Depressive Disorder (MDD). Studies have also shown that clavulanic acid suppresses anxiety and enhances sexual functions in rodent and primate models by a mechanism involving central nervous system (CNS) modulation, although its detailed mechanism of action has yet to be elucidated. To further examine its potential as a CNS modulating agent as well as its mechanism of action, we investigated the effects of clavulanic acid in neuronal cells. Our results indicate that clavulanic acid enhances dopamine release in PC12 and SH-SY5Y cells without affecting dopamine synthesis. Furthermore, using affinity chromatography we were able to identify two proteins, Munc18-1 and Rab4 that potentially bind to clavulanic acid and play a critical role in neurosecretion and the vesicle trafficking process. Consistent with this result, an increase in the translocation of Munc18-1 and Rab4 from the cytoplasm to the plasma membrane was observed in clavulanic acid treated cells. Overall, these data suggest that clavulanic acid enhances dopamine release in a mechanism involving Munc18-1 and Rab4 modulation and warrants further investigation of its therapeutic use in CNS disorders, such as depression.

Advances and scientific progress in psychiatric treatment for mood disorders, such as Major Depressive Disorder (MDD), have shown limitations. MDD affects an estimated 16% of the population [8]. Both genetic and non-genetic factors, such as trauma and stress, can contribute to depression [13]. The focal point of depression research has been directed towards the monoamine hypothesis, which entails the imbalance or deficiency of monoamine neurotransmitters (e.g., dopamine, serotonin, norepinephrine). Although the true cause of depression remains unknown, the introduction of monoamine oxidase inhibitors (MAOIs) and tricyclic antidepressants (TCAs) has supported the monoamine hypothesis. These agents work by enhancing monoamine function [13,14]. The development of selective serotonin reuptake inhibitors (SSRIs) as functional antidepressants has added additional support to the monoamine hypothesis. Nevertheless, current treatments for depression are only effective in less than 50% of patients, indicating a discrepancy in the current knowledge of depression etiology and treatment [16]. Furthermore, antidepressants that alter monoamines have delayed therapeutic benefits, require chronic treatment and often have a variety of undesirable side effects [10]. Therefore, the molecular mechanism of depression may be far more complex and involve multiple signaling pathways regulating neurotransmission.

Interestingly, it was recently reported that all antidepressant treatments increase dopamine in the frontal cortex as well as other areas of the brain through either direct or indirect mechanisms [12]. Lavergne et al. [12] performed an extensive literature search of both chemical and non-chemical antidepressant treatments and found that all antidepressant treatments increase dopamine release in the prefrontal cortex consistently as well as other areas such as the limbic system, nucleus accumbens, striatum and other cortical regions. Therefore, it is unambiguous that an enhancement of dopamine levels is associated with antidepressant treatment.

Clavulanic acid was previously described as a non-competitive inhibitor of β-lactamase and augments other β-lactam family antibiotics, although the compound has negligible intrinsic antibacterial activity [18]. Recent studies have shown that clavulanic acid possesses strong CNS modulating effects. Clavulanic acid decreases anxiety in rodent and primate models [9]. Further findings suggest that clavulanic acid is a neuroprotective agent in Parkinson's models in vivo [5]. Clavulanic acid has also been shown to enhance sexual arousal in animal models, and this effect is hypothesized to be CNS-mediated [1]. Clavulanic acid easily crosses the blood–brain barrier, permitting its viable CNS drug properties. The distribution

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doi:10.1016/j.neulet.2011.09.032

ratio of clavulanic acid between human cerebrospinal fluid and plasma is 0.25, suggesting considerably higher level of brain penetration than most other small molecules [15].

In this report, we investigated the effects of clavulanic acid in two dopaminergic neuronal cell lines. Our data suggests that clavulanic acid enhances dopamine levels in both neuronal cells. We propose that the enhancement of dopamine may be through a mechanism involving vesicle trafficking and fusion through the binding and regulation of Munc18-1 and Rab4.

PC12 cells (adrenal gland; Pheochromocytoma) and SH-SY5Y cells (human neuroblastoma) were obtained from American Type Culture Collection (ATCC, VA). PC12 cells were maintained in poly-D-lysine coated dishes (BD Biocoat, MA) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with heat-inactivated 10% horse serum (HS) (Gibco, MD) and 5% fetal bovine serum (FBS) (Gibco, MD), 100 units/ml penicillin, 100 μg/ml streptomycin in a water-saturated atmosphere of 5% CO_2 at 37 °C. SH-SY5Y cells were cultured in a medium containing DMEM, Hanks' balanced salt solution (HBSS), F-12 medium (2:1:1) with 10% heat inactivated fetal bovine serum (Invitrogen, Carlsbad, CA). SH-SY5Y cells were differentiated to a neuronal phenotype by adding 10 μM of retinoic acid to the culture medium for 3 days; then the media was removed and replaced with fresh media containing 80 nM of 12-O-tetradecanoyl-phorbol-13-acetate (TPA) for another 3 days.

Measurement of dopamine in PC12 and differentiated SH-SY5Y cells was performed using commercially available dopamine ELISA kit (Rocky Mountain Diagnostics, CO). Cells were treated with 100 μM of clavulanic acid for 6 and 12 h. After treatment, cells were harvested and lysed immediately. Cell homogenates were centrifuged at 10,000 × *g* for 20 min at 4 °C and supernatant was used to measure dopamine as per the manufacture's instruction. Protein concentrations were determined using Bradford reagent (Bio-Rad). For dopamine release, cells were treated with 100 μM of clavulanic acid for 6 and12 h and stimulated with 50 mM of K^+ solution for depolarization. Samples were collected and dopamine level was measured immediately as per the manufacture's instruction.

Cells were harvested, lysed and protein concentrations were determined, using the Bradford reagent (Bio-Rad). 25 μg of lysates were resolved on NuPAGE 4–12% Bis–Tris gel (Invitrogen, Carlsbad, CA) followed by Western blotting using the desired antibodies as described [19].

Rat brain tissue (Pel-Freeze Biologicals, AZ) was homogenized in homogenization buffer (60 mM β-glycerophosphate, 15 mM p-nitrophenyl phosphate, 25 mM MOPS (pH 7.2), 15 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 1 mM Na_3VO_4, 1 mM NaF, 1 mM phenyl phosphate, 100 μM benzamidine) with the addition of 1× protease inhibitor at 4 °C. The homogenate was centrifuged at 14,000 rpm for 10 min at 4 °C, and the supernatant was collected and analyzed for total protein concentration by BCA analysis.

Potassium clavulanate (DSM Anti-Infectives, Sweden) was covalently bound to CarboxyLink™ (Immobilized diaminodipropylamine) coupling gel (Pierce, IL) for affinity studies. Brain homogenate was incubated with clavulanic acid conjugated to the activated coupling gel for 2 h with gentle shaking at 4 °C. After washing the gel four times with washing buffer (50 mM Tris–HCl, pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% Nonidet p-40, 100 μM benzamidine, 1× protease inhibitors), bound proteins were released from the affinity gel by the addition of 2× Laemmli sample buffer (BioRad, CA) and heat denaturation at 95 °C for 5 min. The denatured proteins were resolved by 2-dimensional electrophoresis, and protein bands were visualized by staining. Spots that were different from control were excised and identified by protein mass spectrometry sequencing (data not shown). Identified proteins were verified by Western blotting with Munc18-1 or Rab4 antibody (Santa Cruz, CA). To verify specificity of the affinity studies and proteins identified in the 2-dimensional studies, Syntaxin-1 (Santa Cruz) was also used for Western blotting.

For immunofluorescence, differentiated SH-SY5Y cells were grown on coverslips, washed with PBS and fixed for 30 min using 4% paraformaldehyde. The cells were then permeabilized using cold methanol and blocked for 1 h with horse serum (5% in PBS). Fixed cells were incubated with either Munc18-1 or Rab4 antibody at 1:100 dilution, washed and labeled with rhodamine red-linked anti-rabbit secondary antibody (1:100 dilutions). Confocal images were collected using an MRC 1024-krypton/argon laser scanning confocal equipped with a Zeiss LSM 510 Meta photomicroscope.

Data shown in this study were expressed as means ± S.D. Differences between experimental groups were considered significant when $p < 0.05$ by Student's *t*-test. All statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., CA).

Dopamine levels were analyzed in PC12 and differentiated SH-SY5Y cells in the presence or absence of clavulanic acid. Quantitative dopamine levels were measured in both cell lines by enzyme-linked immunosorbent assay. As shown in Fig. 1A, dopamine release was not affected in PC12 cells treated for 6 h with clavulanic acid, but the dopamine level was increased ~1.8 fold in the medium after 12 h of clavulanic acid treatment upon depolarization with K^+. The increase in dopamine by clavulanic acid is attributed to increased release of intracellular dopamine since total amount of dopamine levels remained unchanged from control upon clavulanic acid treatment (Fig. 1A). Additionally, dopamine release was increased ~2 fold and ~2.5 fold in differentiated SH-SY5Y cells treated with clavulanic acid for both 6 and 12 h, respectively. Total amount of intracellular dopamine remained unchanged, indicating that clavulanic acid enhanced release of dopamine after treatment (Fig. 1C). Furthermore, clavulanic acid had no effect on the levels of tyrosine hydroxylase in either cell line (Fig. 1B and D). These results suggest that clavulanic acid does not affect the synthesis of dopamine, but rather increases the release of intracellular dopamine in depolarizing condition.

The following study was performed to identify potential target proteins that bind to clavulanic acid and that are involved in neurotransmitter release. Earlier studies have shown that clavulanic acid does not bind to any well-known signaling receptors, transporters or ion channels involved in neurotransmission [9]. In this study, the eluted fraction of brain homogenate that was mixed with affinity resin alone (no clavulanic acid) or clavulanic acid conjugated affinity resin was analyzed by 2-dimensional gel electrophoresis. Candidate proteins were selected, excised and identified by mass spectrometry. Proteins eluted from the clavulanic acid conjugated affinity resin that were different from control were identified and Munc18-1 and Rab4 were among those binding proteins. Further Western blotting was performed to verify the specificity of Munc18-1 and Rab4 and indicated that both proteins were specifically bound to clavulanic acid (Fig. 2A). Moreover, to show that the affinity binding studies were specific to Munc18-1 and Rab4, Western blots were also probed for Syntaxin-1, a key protein also involved in neurosecretion. Fig. 2B indicates that Syntaxin-1 was not detected in the eluted fraction from the clavulanic acid conjugated resin, indicating that Munc18-1 and Rab4 are specific binding proteins of clavulanic acid.

It is known that Munc18-1 and Rab proteins are essential in the secretion of neurotransmitters from synaptic vesicles. Our binding studies indicate clavulanic acid specifically binds to Munc18-1 and Rab4 and since these proteins play a key role in membrane trafficking and fusion as well as vesicle recycling [3,4,22], we investigated the subcellular localization of Munc18-1 and Rab4 in the presence or absence of clavulanic acid. In SH-SY5Y cells, both Munc18-1 and Rab4 translocated from the cytoplasm to the plasma membrane in