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

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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.

Keywords

Clavulanic acid; dopamine; Rab4/Munc18-1; neurotransmitter release; depression

Introduction

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

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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 [14,13]. 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. 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 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.

Materials and Methods

Cell Culture

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.

Dopamine measurements

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 10,000g 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.

Western blot analyses

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].

Preparation of brain homogenate

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 $MgCl_2$, mM DTT, 1 mM Na_3VO_4, 1 mMNaF, 1 mM phenyl phosphate, 100 μM benzamidine) with the addition of 1X 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.

Target binding affinity studies

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, 1X protease inhibitors), bound proteins were released from the affinity gel by the addition of 2X 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.

Confocal Microscopy

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.

Quantitation of data and statistical analysis

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).

Results

Clavulanic acid enhances dopamine release in neuronal cells

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 Figure 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 (Figure 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 (Figure 1C). Furthermore, clavulanic acid had no effect on the levels of tyrosine hydroxylase in either cell line (Fig 1B and 1D). These results suggest that clavulanic acid does not affect the synthesis of dopamine, but rather increases the release of intracellular dopamine in depolarizing condition.

Identification of possible protein targets of Clavulanic acid

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 (Figure 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. Figure 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.

Clavulanic acid translocates Munc18-1 and Rab4 from the cytoplasm to the membrane

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 the presence of 100 μM clavulanic acid (Figure 3B) compared to vehicle treated control (Figure 3A). It is known that the movement of Rab proteins from the cytoplasm to the plasma membrane is regulated by prenylation, therefore we investigated if clavulanic acid induced translocation of Rab4 affected prenylation. Inhibition of prenylation by simvastatin, a known inhibitor or protein prenylation [17], decreased the effects of clavulanic acid on Rab4 translocation to the plasma membrane, suggesting that proper post-translational modification through prenylation of Rab4 must occur in order for Rab4 to localize to the plasma membrane by clavulanic acid. Overall, these results suggest that both Munc18-1 and Rab4 may be a part of clavulanic acid-induced increase in dopamine release observed in Figure 1.

Discussion

The release of neurotransmitters into the synapse occurs by exocytosis of secretory vesicles. Intracellular membrane fusion events leading to the process of neurotransmitter release are controlled by four classes of proteins: SNARE-proteins, SM-proteins (Sec1/Munc-18 proteins), Rab-proteins and Rab-effectors [6]. It is believed that Rab and Rab-effector proteins regulate and mediate upstream events (vesicle budding, transport, delivery and tethering) and the SNARE- and SM-proteins catalyze the fusion reaction of the secretory vesicle with the plasma membrane.

Among the SM family, Munc18-1 is critical for regulation of vesicle fusion and exocytosis, since deletion of Munc18-1 results in the loss of all synaptic vesicle fusion [25]. Although the exact role of Munc18-1 has yet to be determined it is proposed that Munc18-1 is a key regulator of neurosecretion and that the general function for Munc18-1 and SM proteins is to directly promote SNARE complex assembly [4]. It has been shown that Munc18-1 binds directly to the assembled SNARE complex [2] and can activate SNARE-mediated membrane fusion [20] and fusion between large vesicles and giant membranes [24]. Our findings of clavulanic acid-induced translocation of Munc18-1 to the plasma membrane and of clavulanic acid-induced enhanced release of dopamine from neuronal cells strongly suggest that clavulanic acid-induced dopamine release may be via an interaction with Munc18-1.

The Rab family, regulators of vesicular traffic (reviewed in [23]), consist of more than 60 Rab proteins in mammalian cells. Rab GTPases have been implicated in each step of vesicle formation, trafficking (vesicle budding, transport, delivery and tethering) and SNARE complex formation [21, 26]. Rab proteins switch between their active GTP-bound form which interacts with downstream effector proteins, and their inactive GDP-bound form. In their active GTP-bound form, Rab proteins recruit specific effector proteins onto the cytosolic face of membranes in order to regulate vesicle formation, movement and fusion through their effectors.

Our affinity binding studies showed that clavulanic acid binds to Rab4. Specifically, Rab4 protein has been shown to be associated with early endosomes and regulates “short-loop” (fast) early endosome membrane recycling [22, 7]. Vesicle recycling plays a key role in maintaining homeostasis after vesicle exocytosis. Several mechanisms exist for synaptic vesicle recycling, including fast exocytosis/endocytosis and clathrin-mediated endocytosis. Regardless of the mechanism of endocytosis, all recycled vesicles are loaded with neurotransmitters before subsequent fusion [11]. Early endosomes have been shown to contain domains for Rab4 and Rab5 which are involved in endosome fusion and endocytic recycling. Recycled endosomes contain domains for Rab4 and Rab11 which are necessary for vesicle trafficking from the early endosome to the plasma membrane [23].

Our results of clavulanic acid-induced translocation of Rab4 to the plasma membrane and enhanced dopamine release from the cells suggest that dopamine secretion by clavulanic acid may be due to the interaction of clavulanic acid with Rab4, resulting in subsequent vesicle fusion and potentially endosomal recycling. Rab proteins are initially synthesized in the cytosol where they associate with Rab escort protein (REP) to undergo post-translational modification by the addition of one or two hydrophobic geranylgeranyl groups. This post-translational modification is required to allow for the attachment of the Rab proteins into the lipid bilayer [23]. The modified REP-associated Rab protein in its GDP-bound form is activated to its GTP-bound form upon membrane delivery. This exchange of GDP to GTP is catalyzed by a GDP/GTP exchange factor (GEF) and results in the release of REP. The active membrane bound Rab is then able to carry out its various functions through binding of their effectors [3, 23]. We verified that clavulanic acid was enhancing Rab4 localization to the plasma membrane via the above mechanism through post-translational modification. Upon treatment with simvastatin which is known to inhibit protein prenylation [17], clavulanic acid no longer enhanced Rab4 localization to the plasma membrane suggesting that clavulanic acid enhances dopamine release through a mechanism involving conventional Rab4 post-translational modification in the cytosol and subsequent movement to the plasma membrane.

Recently, it was demonstrated that clavulanic acid, at different effective dose ranges, possesses strong CNS modulating effects, including anti-anxiety effects in rodent and primate models [9], neuroprotective effects in Parkinson's disease models in vivo [5] as well as enhanced sexual arousal in animal models through a proposed CNS-mediated mechanism [1]. These clavulanic acid-induced pharmacological activities may be due to the release of dopamine via clavulanic acid interaction with vesicle trafficking and fusion proteins Munc18-1 and Rab4.

In summary, our current studies show that clavulanic acid enhances dopamine release in two neuronal cell lines. In order to elucidate the mechanism on how clavulanic acid may enhance dopamine levels and possess its strong CNS-modulating effects, we investigated its potential binding partners. Previous receptor based studies showed that clavulanic acid failed to bind to 63 well-known receptors and neurotransmitter-related targets, such as ion channels, second messengers, and other enzymes [9], implying that clavulanic acid acts through a novel mechanism. Therefore we utilized affinity based studies to purify any proteins that may bind to clavulanic acid. Our studies indicate that clavulanic acid binds to Munc18-1 and Rab4 and alters the subcellular localization of both proteins to the plasma membrane in neuronal cells. Indeed, both Munc18-1 and Rab4 protein are critical proteins in vesicle fusion/trafficking and recycling, respectively. We propose that clavulanic acid binds to Munc18-1 and Rab4 enhancing neurosecretion of dopamine. It is possible that other proteins involved in neurosecetion may be altered by clavulanic acid, which have yet to be elucidated; however in our affinity binding studies, clavulanic acid did not bind to Syntaxin-1. Furthermore, since clavulanic acid binds to Munc18-1 and Rab4 and alters the localization of these proteins, it is possible that clavulanic acid may have an effect on the release of other monoamines (5-HT, NA, NE). Previous studies have shown that clavulanic acid enhances the release of both dopamine and 5-HT in vivo [9]. The dual enhancement of both dopamine and 5-HT by clavulanic acid may be a result of enhanced vesicle trafficking and fusion through a mechanism involving Munc18-1 and Rab4. Enhanced vesicle transport and fusion, potentially resulting in increased neurotransmitter release, may prove a novel and ideal therapy in MDD as well as other neurological disorders. Ongoing studies are currently underway to further elucidating the exact mechanism as to how clavulanic acid may bind to Munc18-1 and Rab4 and regulate neurotransmitter release.

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Highlights

- Clavulanic acid, a β-lactamase inhibitor, for the treatment of depression.
- We find clavulanic acid increases dopamine release in two neuronal cell lines.
- Affinity binding studies show clavulanic acid binds to Munc18-1 and Rab4.
- We find clavulanic acid induces localization of Munc18-1/Rab4 to plasma membrane.

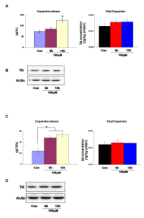

Figure 1. Clavulanic acid enhances dopamine in neuronal cells

(A) PC12 cells were treated with 100 μM clavulanic acid or vehicle control. Dopamine release and total dopamine levels were measured by ELISA at 6 and 12 h post treatment. (B) Tyrosine hydroxylase expression in PC12 cells upon treatment with clavulanic acid for 6 and 12 h was measured. (C) Differentiated SH-SY5Y cells were treated with 100 μM clavulanic acid or vehicle control. Dopamine release and total dopamine levels were measured by ELISA after 6 and 12 h post treatment. (D) Tyrosine hydroxylase expression in differentiated SH-SY5Y upon treatment with clavulanic acid for 6 and 12 h was measured. Values were expressed as mean ± S.D and * indicates values that are significantly different from control ($p<0.05$).

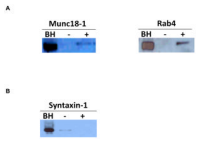

Figure 2. Clavulanic acid binds to Munc18-1 and Rab4

Clavulanic acid conjugated and immobilized onto affinity gel was incubated with rat brain homogenate. Proteins bound to the clavulanic acid conjugated to the gel were eluted and analyzed by 2-dimensional gel electrophoresis and mass spectrometry (data not shown). (A) Munc18-1 and Rab4 were identified as a candidate protein that were present in the elution pool and was verified by western blotting. Brain homogenate alone, proteins eluted from the clavulanic acid conjugated to the affinity gel and proteins eluted from affinity gel alone (primary amine was protected with acetyl group, no clavulanic acid) were run on SDS/PAGE gel. Gels were stained to verify protein loading and then Western blot analysis was performed: 1st lane: brain homogenate (BH) alone probed for the presence of either Munc18-1 or Rab4; 2nd lane (−): control (BH elution pool of resin alone probed for either Munc18-1 or Rab4); 3rd lane (+): indicates the presence of either Munc18-1 or Rab4 from the eluted proteins collected from clavulanic acid conjugated gel. (B) Clavulanic acid does not bind to Syntaxin 1. 1st lane: brain homogenate (BH) alone probed for the presence of Syntaxin-1; 2nd lane (−): control (BH elution pool of resin alone probed for Syntaxin-1); 3rd lane (+): (eluted BH proteins collected from clavulanic acid conjugated affinity resin probed for Syntaxin-1).

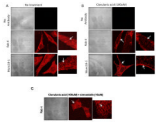

Figure 3. Clavulanic acid enhances the localization of Munc18-1 and Rab4 to the plasma membrane in neuronal cells

SH-SY5Y cells were treated in the absence (A) or presence (B) of 100 μM clavulanic acid for 1 h. Cells were fixed and stained with either Munc18-1 or Rab4 antibody followed by Rhodamine conjugated secondary antibodies. Cells were then visualized at 60X magnification using Zeiss confocal microscope. Corresponding light microscopy pictures were taken as well. White arrows indicate areas of altered Munc18-1 or Rab4 localization (to the plasma membrane). Similarly, cells were also treated with 10 μM simvastatin to inhibit protein prenylation, in the presence of clavulanic acid (C) and stained with Rab4. Images presented here are representative of four independent experiments.