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## RESEARCH ARTICLE

# NEFIRACETAM AND URIDINE ENHANCE THE ACTIVATED PKCε ACTIVITYPOSSIBLY BY LOWERING THE KINASE ACTIVATION ENERGY

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**ABSTRACT**

The pyrrolidinone derivative nefiracetam facilitates hippocampal synaptic transmission in a PKC-dependent manner. The present study was conducted to understand the mechanism underlying the action of nefiracetam on the PKC activity. In the cell-free system, nefiracetam enhanced the activity of PKCε, activated by linoleic acid, in an ATP concentration (2-500 μM)-dependent manner, and a huge enhancement was found at the concentrations higher than 100 μM. Notably, a similar effect was also obtained with uridine. ATP dissolved in water was spontaneously degraded into ADP, AMP, and adenosine in an incubation time (1-15 days)-dependent manner. The ATP degradation was accelerated by adding nefiracetam or uridine. Taken together, the results of the present study show that nefiracetam as well as uridine enhance the activated PKCε activity possibly by assisting ATP hydrolysis and lowering the kinase activation energy.



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## INTRODUCTION

Nefiracetam, a pyrrolidinone derivative developed as an anti-dementia drug, enhances α7 ACh receptor responses in a PKC-dependent manner (Nishizaki *et al*., 1998), thereby causing an increase in presynaptic glutamate release, and then leading to a 'long-term potentiation (LTP)'-like facilitation of hippocampal synaptic transmission (Nishizaki *et al*., 1999; Nishizaki *et al*., 2000a; Nishizaki *et al*., 2000b; Nomura and Nishizaki, 2000). The facilitatory action of nefiracetam was independent of NMDA receptor, that is indispensable for expression of LTP (Matsumoto *et al*., 2002). Like nefiracetam, 2-pyrrolidinone enhances the activated PKCε activity, potentiates α 7 ACh receptor responses, and facilitates hippocampal synaptic transmission (Miyamoto *et al*., 2003). Taken together, the primary site of action of nefiracetam or 2-pyrrolidinone appears to be PKCε. How nefiracetam or 2-pyrrolidinone enhances the PKCε activity, however, remained to be explored. To address this question, the present study assayed the PKCε activity and monitored spontaneous ATP degradation in the cell-free system. Moreover, I speculated that endogenous substances exhibiting an effect similar to nefiracetam might exist. I searched chemical structure analogs of nefiracetam or 2-pyrrolidinone, and chose nucleosides such as cytosine, guanosine, thymidine, and uridine (Figure 1). So, the present study also investigated the effects of the nucleosides on the PKCε activity and spontaneous ATP degradation. The results show that nefiracetam, 2-pyrrolidinone, and uridinemay enhance the activated PKCε activity by assisting ATP hydrolysis and lowering the kinase activation energy.

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## MATERIALS AND METHODS

**Cell-free PKCε assay:** The PKCεactivity was assayed in the cell-free systems by the method as previously described (Kanno *et al*., 2006). Synthetic PKC substrate peptide (Pyr-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu) (Peptide Institute Inc., Osaka, Japan) (10 μM) was reacted with PKCεin a $Ca^{2+}$-free and phosphatidylserine-free medium containing 10 μM linoleic acid, 20 mM Tris-HCl (pH 7.5), 5 mM Mg-acetate, and ATP at concentrations ranging from 2 to 500 μM in the presence and absence of nefiracetam, 2-pyrrolidinone, cytosine, guanosine, thymidine, or uridine at a concentration of 100 μM at 30 °C for 5 min. After loading on a reversed phase HPLC (LC-10ATvp, Shimadzu Co., Kyoto, Japan), a substrate peptide peak and a new product peak, corresponding to non-phosphorylated and phosphorylated peptides, respectively, were detected at an absorbance of 214 nm(SPD-10Avp UV-VIS detector, Shimadzu Co.). The quantity of phosphorylated

substrate peptide (pmol/min) was calculated and used as an index of PKCε activity.

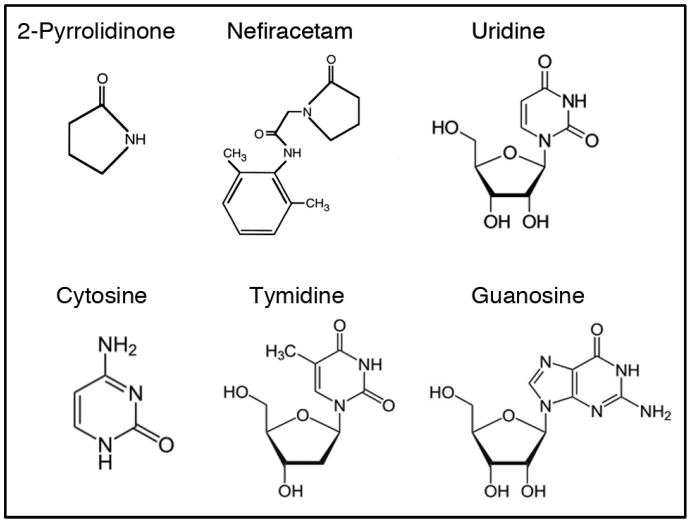


**Figure 1. Chemical structure of 2-pyrrolidinone, nefiracetam, uridine, cytosine, thymidine, and guanosine**

**ATP degradation assay:** ATP dissolved in water (100 μM) was incubated in the presence and absence of nefiracetam, 2-pyrrolididone, cytosine, guanosine, thymidine, or uridineat a concentration of 100 μMfor 10-15 daysat 30 °C. Then, ATP, ADP, AMP, and adenosine were analyzed in the HPLC systems (LC-10ATvp, Shimadzu Co.), and each concentration was quantified using the standard curve of standards in the chromatogram.

**Statistical analysis:** Statistical analysis was carried out using unpaired *t*-test.

## RESULTS

**Nefiracetam, 2-pyrrolidinone, and uridine enhance the activated PKCε activity:** PKC isozymes include conventional PKCs such as PKCα, -βI, -βII, and -γ, novel PKCs such as PKCδ, -ε, -η, -θ, and -μ, and atypical PKCs such as PKCλ/ι for mouse/human, -ζ, and -ν. PKCε, that is preferentially expressed in the presynaptic terminals (Saito *et al*., 1993), is activated by *cis*-unsaturated free fatty acids such as linoleic acid in a $Ca^{2+}$- and diacylglycerol-independent manner (Nishizuka, 1992; Nishizuka, 1995). In the cell-free kinase assay, linoleic acid indeed activated PKCε (Figure 2), but no PKCε activation was inducedby nefiracetam alone in the absence of linoleic acid (data not shown). This implies that nefiracetam does not serve as a direct activator of PKCε. Nefiracetam enhanced the activity of PKCε, activated by linoleic acid, in an ATP concentration (2-500 μM)-dependent manner (Figure 2A). Notably, a robust enhancement was found at the ATP concentrations higher than 100 μM, reaching approximately 140 folds of the basal levels at 500 μM (Figure 2A). Nefiracetam is a pyrrolidinone derivative. 2-Pyrrolidinone also enhanced the activated PKCε activity, although the degree was much smaller than that for nefiracetam (Figure 2B). The next attempt was to explore the effect of the nucleosides cytosine, guanosine, thymidine, and uridine on the PKCε activity. Intriguingly, uridine enhanced the activated PKCε activity in a fashion that mimics the effect of nefiracetam, the degree reaching approximately 120 foldsof the basal levels at 500 μM (Figure 2C). In contrast, no enhancement in the activated PKCε activity was obtained with the other nucleosides examined here guanosine (Figure 2D), cytosine, and thymidine (data not shown). This explains that the effect of uridine on the PKCε activity is not due to the non-specific action of nucleosides.

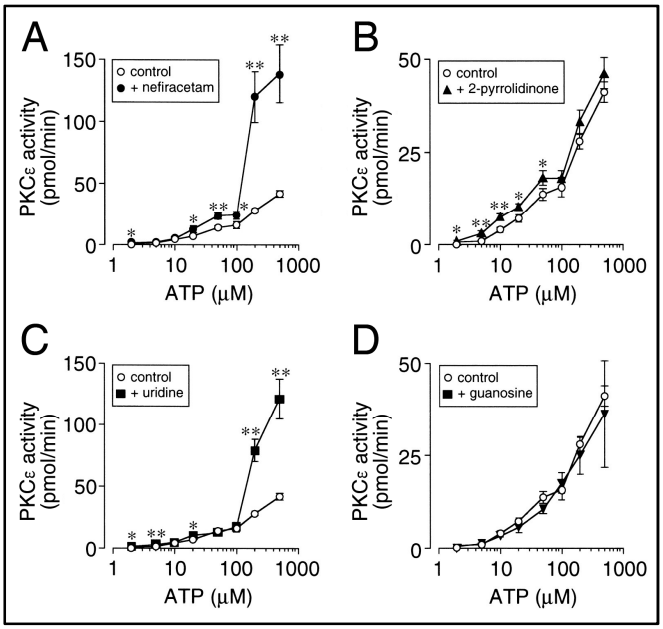


**Figure 2. PKCε activity. In the cell-free system, synthetic PKC substrate peptide (10 μM) was reacted with PKCεin a $Ca^{2+}$-free and phosphatidylserine-free medium containing linoleic acid (10 μM) and ATP at concentrations as indicated in the presence and absence of nefiracetam (100 μM) (A), 2-pyrrolidinone (100 μM) (B), uridine (100 μM) (C), or guanosine (100 μM) (D) at 30 °C for 5 min, and the quantity of phosphorylated substrate peptide was calculated. In the graphs, each point represents the mean (± SEM)PKCε activity (pmol/min) (n=6 independent experiments). *$P$<0.01, **$P$<0.001; unpaired *t*-test.**

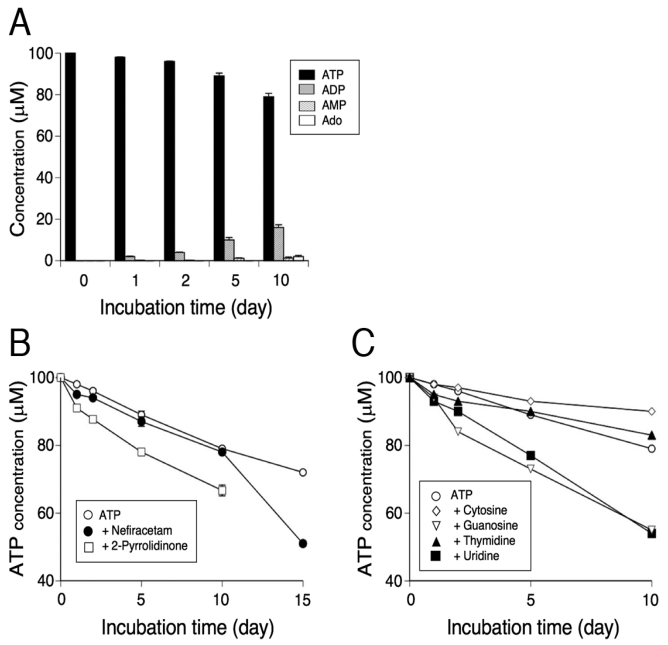


**Figure 3. Spontaneous ATP degradation.(A) ATP dissolved inwater (100 μM)was incubated for 10 daysat 30 °C, followed by quantification of ATP, ADP, AMP, and adenosine. In the graph, each column represents the mean (± SEM) concentration(μM)(n=3 independent experiments). (B)(C) In a different set of experiments, ATP dissolved inwater (100 μM) was incubated in the absence and presence of nefiracetam, 2-pyrrolididone, cytosine, guanosine, thymidine,or uridineat a concentration of 100 μMfor 10-15 daysat 30 °C, followed by quantification of ATP. In the graphs, each point represents the mean (± SEM) ATP concentration (μM)(n=3 independent experiments).**

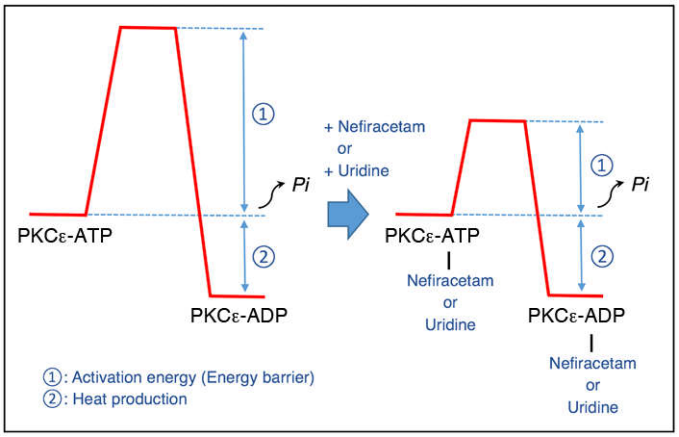


**Figure 4. A hypothetic diagram for the action of nefiracetam or uridine on PKCε activation.Nefiracetam or uridine lowers the PKCε activation energy (energy barrier) by assistinghydrolysis of ATP, that binds to PKCε, leading to an enhancement in the activated PKCε activity.**

**Nefiracetam, 2-pyrrolidinone, and uridine accelerate spontaneous ATP degradation:** When ATP dissolved in water was incubatedat 30 °C, the concentration of ATP decreased in an incubation time (1-10 day)-dependent manner, and in parallel with it the concentrations of ADP, AMP, and adenosine gradually went up (Figure 3A). This implies that ATP is spontaneously degraded into ADP, AMP, and adenosine.Co-incubation of ATP and nefiracetam accelerated spontaneous ATP degradation, and the prominent effect was obtained after 10-day incubation (Figure 3B).Co-incubation of ATP and 2-pyrrolidinone decreased the ATP concentration apparently from 1 day after incubation (Figure 3B).Likewise, co-incubation of ATP and uridine accelerated spontaneous ATP degradation from 1 day after incubation (Figure 3C). A similar effect was obtained with guanosine, but cytosine and thymidine had no effect on the ATP degradation (Figure 3C).

## DISCUSSION

The results of the present study clearly demonstrate that nefiracetam has the potential to enhance the activated PKCε activity. 2-Pyrrolidinone also enhanced the activated PKCε activity, but to an extent lesser than nefiracetam. This suggests that 5-membered ring lactam structure is critical for the nefiracetam action. The most striking finding is that uridine, whereas other nucleosides examined here had no effect,exhibited an effect similar to nefiracetam on the activated PKCε activity. PKCε was not activated by nefiracetam alone or uridine alone. Then, the question addressing is how nefiracetam or uridine enhances the activated PKCε activity. PKCε is inactivated in the closed conformation at the hinge domain, and when the closed conformation is opened by binding phosphatidylserine, diacylglycerol, or *cis*-unsaturated free fatty acids, PKCε becomes the active form. Subsequently, ATP binds to the active form of PKCε, and ATP is hydrolyzed into ADP, to producephosphate, that is transferred to the substrate, i.e., phosphorylation.I postulated that if ATP hydrolysis is accelerated, the PKCε activity should be enhanced. To address this hypothesis, I examined the effect of nefiracetam on ATP degradation. Amazingly, nefiracetam as well as 2-pyrrolidinone accelerated spontaneous ATP degradation. A similar effect was also obtained with uridine and guanosine. Overall, these results raise the possibility that nefiracetam, 2-pyrrolidinone, and uridine might reducethe PKCε activation energy (energy barrier) by assisting ATP hydrolysis, causing an enhancement inthe activated PKCε activity (Figure 4). Why guanosine, in spite of acceleration of spontaneous ATP degradation, had no effect on the activated PKCε activity, however, remains an open question.PKCε, that is rich in the presynaptic terminals (Saito *et al*., 1993), stimulates vesicular exocytosis of α7 ACh receptor and increases cell surface localization of the receptor (Kanno *et al*., 2012), causing an increase in presynaptic glutamate release (Shimizu *et al*., 2011), and then leasing to facilitation of hippocampal synaptic transmission (Nishizaki *et al*., 2000a; Nishizaki *et al*., 2000b; Yamamoto *et al*., 2005). The primary site of action of nefiracetam and 2-pyrrolidinone on synaptic transmission, therefore, would be PKCε. Uridine is shown to improve cognitive functions by co-application with the *cis*-unsaturated free fatty acid docosahexaenoic acid (DHA) (Holguin *et al*., 2008). The effect of uridine is thought to be caused by increasing synaptic proteins, phospholipids, and CDP-choline (Holguin *et al*., 2008; Ulus *et al*., 2006; Wurtman *et al*., 2006). In addition to these factors, the results of the present study indicate that PKCε may contribute to the effect of uridine on cognitive functions; in other words, uridine may enhance the activity of PKCε, activated by DHA, which triggers facilitation of hippocampal synaptic transmission. This also suggests that uridine acts as an endogenous cognitive enhancer by targeting PKCε.

### Conclusion

In the present study, nefiracetam, 2-pyrrolidinone, and uridine accelerated spontaneous ATP degradation and enhanced the activity of PKCε, activated by linoleic acid, in an ATP concentration (2-500 μM)-dependent manner. This indicates that nefiracetam, 2-pyrrolidinone, and uridine might reduce the PKCε activation energy (energy barrier) by assisting ATP hydrolysis, providing the novel regulatory mechanism of PKCεactivation.

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