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European Journal of Pharmacology 449 (2002) 85–90

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Interaction of the neurosteroid alphaxalone with conventional antiepileptic drugs in different types of experimental seizures

Kinga K. Borowicz [a], Marek Zadrożniak [a], Mariusz Świąder [b], Aneta Kowalska [b], Zdzisław Kleinrok [b], Stanisław J. Czuczwar [a,c,*]

[a] *Department of Pathophysiology, Medical University, Jaczewskiego 8, 20-090 Lublin, Poland*
[b] *Department of Pharmacology and Toxicology, Medical University, Jaczewskiego 8, 20-090 Lublin, Poland*
[c] *Isotope Laboratory, Institute of Agricultural Medicine, Jaczewskiego 2, 20-950 Lublin, Poland*

Received 31 January 2002; received in revised form 6 June 2002; accepted 11 June 2002

Abstract

A number of neurosteroids exert antiseizure and/or neuroprotective properties. The aim of this study was to evaluate the effect of the neurosteroid alphaxalone on the protective action of conventional antiepileptics in four seizure tests. Alphaxalone (up to 5 mg/kg) did not exert a significant action against amygdala-kindled seizures in rats, or against pentetrazole- or aminophylline-induced convulsions in mice. The neuroactive steroid at the dose of 2.5 mg/kg significantly raised the threshold for electroconvulsions in mice. At 2.5 mg/kg, alphaxalone diminished the protective activity of valproate against maximal electroshock and at 2.5–5 mg/kg against pentetrazole-induced seizures in mice. However, alphaxalone (2.5 mg/kg) did not affect the protective activity of carbamazepine, diphenylhydantoin, phenobarbital or clonazepam against maximal electroshock and at 5 mg/kg did not affect that of phenobarbital, clonazepam and ethosuximide against pentetrazole-induced convulsions. Insignificant results were also obtained in the case of co-administration of alphaxalone with phenobarbital, valproate, clonazepam and carbamazepine against aminophylline-evoked seizures in mice. Also, in the kindling model of epilepsy, combinations of the neuroactive steroid (2.5 mg/kg) with valproate, carbamazepine, phenobarbital, diphenylhydantoin or clonazepam at their subprotective doses did not result in pro- or anticonvulsant activity. Valproate (284 mg/kg; the dose used in combination with alphaxalone) produced significant memory deficits in mice. Alphaxalone (2.5 mg/kg), valproate (at its ED_{50} value of 226 mg/kg) and the combination of valproate (284 mg/kg) with alphaxalone (2.5 mg/kg) did not affect long-term memory, evaluated in the passive avoidance task with mice. Alphaxalone administered alone or in combination with valproate caused no motor impairment in experimental animals. Finally, alphaxalone (2.5 and 5 mg/kg) significantly increased the free plasma levels of valproate, strongly indicating that the neuroactive steroid-induced reduction of the protective activity of valproate is not related to pharmacokinetic phenomena. Summing up, alphaxalone does not seem to be a promising candidate for adjunctive treatment of epilepsy.

Keywords: Alphaxalone; Antiepileptic drug; Amygdala-kindled seizure; Pentetrazole; Aminophylline; Electroshock maximal

1. Introduction

Epilepsy continues to be a significant clinical problem as current medications neither adequately control seizures nor are free of untoward side effects. Neuroactive steroids are naturally occurring or synthetically derived compounds, many of which have anticonvulsant, anesthetic, anxiolytic, analgesic or hypnotic properties (Beekman et al., 1998). Modulation of the neuroactive steroid site on the γ-aminobutyric acid ($GABA_A$) receptor complex may be an important new approach for pharmaceutical interventions in epilepsy. Consistent with their GABA-ergic actions, the neuroactive steroids as well as diazepam and phenobarbital dose dependently protect against clonic convulsions induced by pentetrazole. Moreover, in contrast to diazepam and phenobarbital, all the neuroactive steroids produce full protection against cocaine-induced convulsions (Gasior et al., 1999). Some of neurosteroids are effective against the seizures induced by *N*-methyl-D-aspartate (NMDA; Budziszewska et al., 1998; Gasior et al., 1997). The distinct profile of anticonvulsant activity of the neuroactive steroids may be

* Corresponding author. Department of Pathophysiology, Medical University, Jaczewskiego 8, 20-090 Lublin, Poland. Tel.: +48-81-742-5837; fax: +48-81-742-5828.
E-mail address: czuczwar@galen.imw.lublin.pl (S.J. Czuczwar).

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PII: S0014-2999(02)01975-1

related to their combined actions on GABA and NMDA-mediated events or voltage-operated Ca^{2+} channels. These results help to define the neuroactive steroids as a novel class of anticonvulsant agents and suggest their potential in clinical practice (Gasior et al., 1997, 1999). Ganaxolone (3-α-hydroxy-3-α-methyl-5-α-pregnan-20-one) is an efficacious anticonvulsant agent against a variety of acute seizures, as well as in electrical and chemical kindling models, and is currently under phase II clinical investigation for epilepsy (Beekman et al., 1998). In fact, efficacy has been reported in preclinical models of epilepsy, anxiety, insomnia, migraine and drug dependence. Clinical evidence to date is generally supportive of these findings and indicates that neuroactive steroids are generally well tolerated. Taken as a whole, current data suggest that neuroactive steroids could have a future role in clinical practice (Gasior et al., 1999).

Alphaxalone, also a neurosteroid compound, is a positive modulator of $GABA_A$ receptors (Park-Chung et al., 1999). A mixture of alphaxalone and alphadolone, used for "steroid anesthesia", exerts an anticonvulsive action against pentetrazole-evoked seizures and maximal electroshock-induced seizures in rats. However, the mixture administered at its protective doses also produces severe neurological adverse effects (Peterson, 1989).

In view of the facts given above, we decided to evaluate the interaction between alphaxalone and conventional antiepileptic drugs in different seizure models—maximal electroshock-, pentetrazole-, aminophylline-induced convulsions in mice and amygdala-kindled seizures in rats. Enhancement of the anticonvulsant activity of some antiepileptic drugs could result in experimental clues for combination therapy in epilepsy, which is essential for about 30% of epileptic patients (Deckers et al., 2000). Moreover, inhibition of aminophylline-induced convulsions by a combination of an antiepileptic + alphaxalone could suggest a possible therapeutic procedure for the management of aminophylline overdose.

2. Materials and methods

2.1. Animals and experimental conditions

Male Wistar rats (200–250 g) and male Swiss mice (20–25 g) were used throughout the experiments. They were housed under standard laboratory conditions. All experiments were done at the same time (between 9.00 and 13.00) to minimize circadian influences on seizure susceptibility. The experimental groups consisted of eight rats or mice. The experimental procedures run in this study were approved by the Lublin Bioethical Committee.

2.2. Surgery and kindling procedure

The rats were anesthetized with pentobarbital (50 mg/kg i.p.) and received stereotaxic implantation of one bipolar electrode in the right basolateral amygdala (Paxinos and Watson, 1986).

After a post-operative period of 2 weeks, stimulation of the amygdala was initiated. Each stimulus consisted of a 1-s train of 50 Hz, 1-ms biphasic square-wave pulses, with pulse amplitude of 500 μA, and was delivered every 24 h, until at least 10 sequential fully kindled stage 5 seizures were elicited. The seizure severity was assessed according to a modification of Racine's system on a scale from 1 to 5 points (Racine, 1972). The procedure in detail has been recently published elsewhere (Borowicz et al., 2002).

2.3. Electroconvulsions

Electroconvulsions in mice were produced with ear-clip electrodes and alternating current delivered by a Hugo Sachs (Type 221, Freiburg, Germany) generator. The stimulus duration was 0.2 s. Full tonic extension of both hind limbs was taken as the endpoint. The convulsive threshold was evaluated as CS_{50}, which is the current strength (in mA) required to produce tonic hind limb extension in 50% of the animals tested. To calculate the convulsive threshold, at least three groups of mice (consisting of at least eight animals per group) were challenged with electroshocks of various intensities. An intensity–response curve was calculated with a computer, based on the percentage of animals convulsing in experimental groups. To calculate the ED_{50} value for the respective drug, the mice were challenged with maximal electroshock (25 mA) which exceeded more than three-fold the CS_{50} value of 7.7 mA. At least four groups (eight animals per group) were used to estimate each ED_{50} value.

2.4. Pentetrazole- and aminophylline-induced seizures

At least four groups of mice (eight mice per group) were injected with various doses of an antiepileptic and with pentetrazole (85 mg/kg) or aminophylline (300 mg/kg). The doses in parentheses are the 97% convulsant doses (CD_{97}s), at which each convulsant was expected to produce clonic seizures in 97% of mice. Following injection, animals were placed separately in transparent Plexiglass cages (25 × 15 × 10 cm) and observed for 30 min (in the case of pentetrazole) or 60 min (in the case of aminophylline) for the occurrence of clonic seizures. Clonic seizure activity was defined as clonus of whole body lasting over 3 s, with an accompanying loss of the righting reflex. To estimate the respective ED_{50} values for antiepileptic drugs, an intensity–response curve was calculated on the basis of the percentage of mice with clonic convulsions.

2.5. Drugs

Alphaxalone (RBI, Natick, MA, USA), clonazepam (Polfa, Warsaw, Poland), diphenylhydantoin, carbamaze-

pine, and ethosuximide (all three drugs purchased from Sigma, St. Louis, MO, USA) were suspended in a 1% solution of Tween 80 (Sigma). Valproate (Polfa, Rzeszów, Poland), phenobarbital (Polfa, Warsaw, Poland), and two convulsants, pentetrazole and aminophylline (both from Sigma), were brought into solution with sterile saline. Except for pentetrazole, which was administered s.c. in a volume of 5 ml/kg, all the remaining agents were given i.p. in a volume of 10 ml/kg. Diphenylhydantoin was administered 120 min, phenobarbital 60 min, valproate, carbamazepine and clonazepam 30 min, and alphaxalone 15 min before tests.

2.6. Passive avoidance task

The rats were placed in an illuminated box (40 × 40 × 30 cm) connected to a dark box (40 × 40 × 30 cm), which was equipped with an electric grid floor. The respective dimensions of the boxes for mice were 10 × 13 × 15 and 25 × 20 × 15 cm. Entrance to the dark box was punished by administering an electric footshock of 2-s duration and intensity of 0.7 mA for rats and 0.6 mA for mice. The animals that did not enter the dark compartment were excluded from the experiment. On the next day (24 h later), the same animals were put into the illuminated box and observed for up to 180 s. The time period an animal entered the dark box was subsequently noted and the medians with 25 and 75 percentiles were calculated. According to Venault et al. (1986), the step-through passive avoidance task is a measure of long-term memory. Drugs or drug combinations were administered only on the first day of the experiment.

2.7. Rotorod test

Motor coordination was assessed with the use of the rotorod test (Dunham and Miya, 1957). Each animal was placed on a 4-cm (for mice) and 6-cm (for rats) diameter rod rotating at 6 rev/min. The time until an animal fell off was noted and the test was run up to 120 s. The results are expressed in the form of medians with 25 and 75 percentiles.

2.8. Estimation of the free plasma concentration of valproate

The animals were given valproate + saline or valproate + alphaxalone (2.5 mg/kg). Blood samples of approximately 1 ml were collected into Eppendorf tubes. Samples of blood were centrifuged at 10 000 rpm (Abbott centrifuge, Irving, TX, USA) for 3 min and plasma samples of 200 μl were pipetted into a micropartition system, MPS-1 (Amicon, Danvers, MA, USA), for the separation of free from protein-bound microsolutes. Then, the MPS-1 tubes were centrifuged at 3000 rpm (MPW-360 centrifuge; Mechanika Precyzyjna, Warsaw, Poland) for 10 min and 50-μl filtrate samples were pipetted into original Abbott system cartridges, which were subsequently put into a carousel for up to 20 samples. Control plasma samples of valproate were placed at the beginning and end of each carousel for verification of the calibration. The free plasma concentration of valproate was estimated by immunofluorescence, with an Abbott TDx analyzer (Abbott). Plasma concentration is expressed in μg/ml as means ± S.D. of eight determinations.

2.9. Statistics

The statistical significance of differences between seizure scores in kindled rats was calculated by the Wilcoxon signed rank test. Seizure and afterdischarge durations in these animals were compared with Student's *t*-test for paired data. Kruskal–Wallis analysis followed by posthoc Dunn's test was used to analyze the results obtained in the passive avoidance and rotorod tasks. ED_{50} values and statistical analysis of the results obtained in the electroconvulsive, pentetrazole and aminophylline tests were calculated by computer probit analysis, according to Litchfield and Wilcoxon (1949). Plasma concentrations of valproate alone or in combination with alphaxalone were evaluated with unpaired Student's *t*-test.

3. Results

3.1. Effects of alphaxalone on amygdala-kindled seizures and on the protective action of antiepileptics in fully kindled rats

Alphaxalone (up to 5.0 mg/kg) did not influence afterdischarge threshold, seizure severity, seizure duration, or afterdischarge duration. Also, co-administration of alphaxalone (2.5 mg/kg) with subprotective doses of valproate (50 mg/kg), carbamazepine (15 mg/kg), phenobarbital (15 mg/kg), diphenylhydantoin (2.5 mg/kg), or clonazepam (0.01 mg/kg), did not affect any seizure parameter in rats (results not shown in the tables).

3.2. Effects of alphaxalone on the protective activity of antiepileptics against maximal electroshock-induced seizures in mice

Alphaxalone (2.5 mg/kg) significantly raised the threshold for electroconvulsions from 7.7 (6.6–8.8) to 10.3 (8.2–12.9) mA. The neuroactive steroid (2.5 mg/kg) did not significantly affect the ED_{50} values of phenobarbital (18.5 mg/kg), diphenylhydantoin (11.7 mg/kg), carbamazepine (10.4 mg/kg), or clonazepam (14.4 mg/kg) against maximal electroshock-induced seizures in mice (results not shown in the tables). However, alphaxalone (2.5 mg/kg) significantly impaired the protective activity of valproate, increasing its ED_{50} value from 226 to 284 mg/kg. The anticonvulsant action of this antiepileptic drug was not modified by the neuroactive steroid given at 1.25 mg/kg (Table 1).

Table 1
Influence of alphaxalone on the anticonvulsant activity of valproate against maximal electroshock (MES)-, pentetrazole (PTZ)-, and aminophylline (AMI)-induced seizures in mice

Treatment (mg/kg)	ED_{50} (MES)	ED_{50} (PTZ)	ED_{50} (AMI)
VPA + saline	226 (201–255)	143 (119–172)	391 (316–484)
VPA + AXL (1.25)	244 (221–269)	174 (154–196)	ND
VPA + AXL (2.5)	284 (262–308) *	199 (179–221) *	445 (350–567)
VPA + AXL (5)	ND	193 (174–218) *	428 (364–518)

The data are given as 50% effective doses (ED_{50}s; 95% confidence limits) for the protection against MES-, PTZ- and AMI-induced seizures. VPA, valproate; AXL, alphaxalone; ND, not determined.
* $P < 0.01$ vs. respective control group.

3.3. Effect of alphaxalone on the protective action of antiepileptics in pentetrazole-induced seizures in mice

Alphaxalone (up to 5 mg/kg) was not effective against pentetrazole-evoked convulsions in mice and did not influence the ED_{50} values of phenobarbital (7.6 mg/kg), clonazepam (0.03 mg/kg) or ethosuximide (124 mg/kg) in this model of experimental epilepsy. However, the neurosteroid (2.5 and 5 mg/kg) significantly reduced the protective action of valproate, increasing its ED_{50} value from 143 to 199 and 193 mg/kg, respectively. The anticonvulsant activity of valproate was not affected by alphaxalone at 1.25 mg/kg (Table 1).

3.4. Effect of alphaxalone on the protective action of antiepileptics in aminophylline-induced seizures in mice

Alphaxalone (up to 5 mg/kg) was ineffective per se against aminophylline-induced seizures and did not change the ED_{50} values of valproate (391 mg/kg; Table 1), clonazepam (4.5 mg/kg), or phenobarbital (64.6 mg/kg; results not shown in the tables).

Table 2
Influence of alphaxalone and valproate on long-term memory and motor impairment in mice

Treatment (mg/kg)	Retention time (s)	Rotorod task (s)
Vehicle	180 (180; 180)	120 (120; 120)
AXL (2.5)	180 (180; 180)	120 (120; 120)
AXL (5)	168 (143; 180)	81 (69; 98) *
VPA (226)	129 (73; 180) *	120 (120; 120)
VPA (284)	116 (68; 139) *	104 (92; 120)
VPA (284) + AXL (2.5)	180 (68; 180) * *	120 (120, 120)
VPA (193)	151 (137; 180) * * *	120 (120; 120)
VPA (193) + AXL (5)	155 (124; 180)	72 (54; 103) *

Table data are given as median values (with 25, 75 percentiles) of six determinations. Motor impairment was indicated when the animals were unable to perform the rotorod task within 120 s. Long-term memory of the animal was considered when it did not avoid the dark compartment within 180 s (see Materials and methods). Kruskal–Wallis test followed by Dunn's posthoc test were used for statistical analysis of the data. For abbreviations, see also the legend of Table 1.
* $P < 0.01$ vs. respective vehicle-treated group.
** $P < 0.05$ vs. VPA (284 mg/kg)-treated group.
*** $P < 0.05$ vs. respective vehicle-treated group.

Table 3
Influence of alphaxalone on the free plasma concentration of valproate

Treatment (mg/kg)	Free plasma concentration (μg/ml)
VPA (284) + saline	158.4 ± 14.3
VPA (284) + AXL (2.5)	172.6 ± 16.8 *
VPA (193)	126.5 ± 13.1
VPA (193) + AXL (5)	149.8 ± 15.4 *

Values are given as means (in μg/ml) of eight determinations in mice. Unpaired Student's *t*-test was used for statistical analysis of free plasma levels of valproate. For abbreviations, see the legend to Table 1.
* $P < 0.05$ vs. control group.

3.5. Passive avoidance task

The saline-treated animals did not enter the dark box within the observation time limit (180 s). Alphaxalone (2.5 and 5 mg/kg) did not produce any significant impairment of long-term memory in rats (resuts not shown in the tables) or mice (Table 2). Valproate administered alone at its ED_{50} dose against maximal electroshock and pentetrazole or doses used for combinations with the neurosteroid (193 and 284 mg/kg, respectively) significantly reduced retention in mice. However, combinations of this antiepileptic with alphaxalone did not disturb long-term memory in mice (Table 2).

3.6. Rotorod test

Valproate alone (193–284 mg/kg) was ineffective in the rotorod test. Alphaxalone (2.5 mg/kg), or its combination with valproate (284 mg/kg), did not disturb motor coordination in mice, either. Conversely, alphaxalone (5 mg/kg) either alone or co-administered with valproate (193 mg/kg) caused significant motor impairment (Table 2).

3.7. Effect of alphaxalone on the free plasma levels of antiepileptic drugs

Alphaxalone (2.5 and 5 mg/kg) significantly raised the free plasma concentration of valproate (284 or 193 mg/kg) from 158.4 to 172.6 μg/ml and from 126.5 to 149.8 μg/ml, respectively (Table 3).

4. Discussion

Our results demonstrate that alphaxalone (2.5 mg/kg) exerted protective activity in the electroconvulsive threshold test and, unexpectedly, it significantly reduced the anticonvulsant action of valproate against maximal electroshock- and pentetrazole-induced seizures in mice. However, the neurosteroid remained ineffective against the effect of valproate on aminophylline-induced convulsions in mice, or amygdala-kindled seizures in rats. Other antiepileptics

were not affected by alphaxalone in the four seizure models used. It is interesting that alphaxalone increased the free plasma concentration of valproate, which may further indicate that the neurosteroid-induced reduction of the protective activity of valproate is solely dependent on a pharmacodynamic mechanism, clearly overcoming the pharmacokinetic interaction.

The lack of interaction between alphaxalone and most of the conventional antiepileptics is rather unexpected. It appears that the $GABA_A$/benzodiazepine receptor complex is a target of action for neurosteroids as well as for many antiepileptic drugs (Brot et al., 1995). It was also reported that anesthesia induction in male rats required about four-fold more alphaxalone/alphadolone mixture than in females. This sex difference was age dependent and seemed to result from markedly higher levels of progesterone in female animals (Fink et al., 1982). Progesterone has proven to be a positive modulator of the $GABA_A$ receptor complex (Mohammad et al., 1998). According to Johnstone and Bancroft (1988), alphaxalone significantly increased androstenedione concentrations in cats. It is noteworthy that androstenedione may be a positive modulator of $GABA_A$, or a negative modulator of NMDA receptors (Herzog, 1999). All the above data may, at least partially, explain the nature of the protective action of alphaxalone in the electroconvulsive threshold test. However, it was also reported that alphaxalone produced excitation of a single isolated neuron (MacIver and Roth, 1987) and prolonged the decay of evoked inhibitory postsynaptic currents (Harrison et al., 1987). This may be of some importance in respect of the neurosteroid-evoked reduction of the protective activity of valproate against maximal electroshock- and pentetrazole-induced seizures. It is evident that alphaxalone allosterically regulated GABA-ergic transmission, and in relatively high concentrations inhibited neuronal $\alpha_4\beta_2$ heteromeric and α_7 homomeric nicotinic receptors (Lambert et al., 2001). In contrast, the neurosteroid at its effective concentrations did not bind to glycine, AMPA, NMDA or $5\text{-}HT_3$ receptors (Lambert et al., 2001). It is quite likely that alphaxalone, like several neurosteroids (Budziszewska et al., 1998), acts as an allosteric positive or negative modulator of the $GABA_A$ receptor complex, which would depend on its dose regimen and interactions with other drugs.

Since valproate is the only drug among the conventional antiepileptics that increases GABA concentrations in the synaptic cleft (Mehta and Ticku, 1999), the hypothesis arises that alphaxalone might block GABA binding sites within the postsynaptic $GABA_A$ receptor complex. This could partially explain the alphaxalone-induced attenuation of the protective action of valproate against maximal electroshock and pentetrazole seizure tests in mice. The nature of this phenomenon might be also associated with an unknown mechanism of action of alphaxalone or valproate, other than their influence on GABA-ergic transmission, Na^+ channels conductance, or NMDA-mediated events in the central nervous system (Steppuhn and Turski, 1993). One hypothetical reason for aminophylline-induced convulsions is that in this seizure test the GABA-ergic agonists muscimol and aminooxyacetic acid are actually proconvulsant (Stone and Javid, 1980, 1981). In this context, valproate has to provide protection in this test via mechanisms independent of GABA-mediated events. The lack of interaction between alphaxalone and valproate in amygdala-kindled seizures remains unclear.

In summary, alphaxalone does not seem to be a promising candidate for the adjunctive treatment of epilepsy. Since maximal electroshock- and pentetrazole-induced convulsions are experimental models of human generalized tonic–clonic and myoclonic seizures (Löscher and Schmidt, 1988), alphaxalone might be expected to reduce the protection offered by valproate in such cases. This is doubly disappointing in view of the fact that the neurosteroid could decrease some adverse effects of conventional antiepileptic drugs. As we have shown, considering long-term memory impairment, combined treatment with valproate and alphaxalone (providing a 50% protection against seizures) was superior to treatment with valproate administered alone at its ED_{50} dose. However, alphaxalone (5 mg/kg) in combination with valproate impaired motor coordination. Interestingly, alphaxalone in a dose of 18 mg/kg was reported to enhance the anticonvulsant activity of flurazepam against electroconvulsions in mice (Deutsch et al., 1996). In the present study, alphaxalone has raised the electroconvulsive threshold at 2.5 mg/kg and at 5 mg/kg impaired motor coordination in the rotorod test. This is why the neuroactive steroid was not tested in higher doses in this study.

The present findings cannot be generalized to all neurosteroids. For instance, generally Ca^{2+} channel inhibitors potentiate the protective activity of conventional antiepileptic drugs against maximal electroshock (Czuczwar et al., 1990, 1992), although niguldipine, while it increased the threshold for electroconvulsions, reduced the anticonvulsant action of carbamazepine and diphenylhydantoin (Borowicz et al., 1997). Alphaxalone, like niguldipine among Ca^{2+} channel inhibitors, might be a negative modulator of the protective efficacy of some antiepileptic drugs. Actually, another neurosteroid, ganaxolone, very potently potentiates the anticonvulsant activity of diazepam against pentetrazole-induced seizure activity in mice (Gasior et al., 1997).

Acknowledgements

This study was supported by Janssen-Cilag (Div. Johnson and Johnson, Warsaw, Poland) and Lublin Medical University.

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