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Original article ■

Involvement of the Vanilloid Receptor 1 in the Mechanisms of Analgesic Effect of Amizonum

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SUMMARY

The study of features of pharmacodynamics of a new analgesic is an important and urgent task of modern pharmacology. These data allow us to clarify the nosology for application of an analgesic and to create a theoretical background to optimize its use. An effect mediated by the transient receptor potential cation channel, subfamily V, member 1 (TRPV1) activation can also be an effective mechanism of the analgesic action. We evaluated the possibility of TRPV1 participation in implementation of the analgesic effect with the antiviral action of amizonum during the experiment. It is known that amino acids Tyr511 and Ser512 are the main components of the active site of TRPV1. In this connection, dipeptide Tyr-Ser has been completely synthesized as a model of the active site of TRPV1. In the experiment model this was shown, using the spectrophotometric method, with the formation of the "capsaicin - Tyr-Ser" intermolecular complex at the level of the stability constant K_{kor}=0.998 and K_r=0.3•10^{-4} L/mol and the "amizonum - Tyr-Ser" weak intermolecular complex Kr=0.05•10^4 L/mol; K_{kor}= 0.995, respectively. The data verification was carried out in experiments in vitro (isolated rat-portal vein) and in vivo (Tail-flick model), with the TRPV1 agonist. It was shown that the amplitude of smooth muscle (SM) contraction of the portal vein at a capsaicin concentration 0.1 μmol/L, 0.5 μmol/L capsazepine, and 1.0 μmol/L amizonum was +30.3±5.3%, -3.2± 2.7% and +7.1±3.2% from initial level, respectivelly. In a combined application of amizonum with capsaicin or capsazepine, the amplitude of contraction of the SM portal vein was 20.1± 1.3% and -3.0±1.4%, respectively. This indicates the absence of action of amizonum under combined use of capsaicinoids. The Tail-flick model showed atypical potentiation of the amizonum antinociception with the use of capsaicin. The obtained data suggest the low probability of the participation of TRPV1 in the implementation of the antinociceptive action of amizonum.

Key words: **amizonum, capsaicin, capsazepine, dipeptide Tyr-Ser (Tyr-Ser), TRPV1**

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INTRODUCTION

In the last decade in connection with the discovery of a significant number of different types of specific receptors located on nociceptive neurons (tetrodotoxin-resistant sodium channels, purinergic receptor RH3, the transient receptor potential vanilloid receptor 1 (TRPV1), etc., special attention is given to the receptor action, both in the development of new analgesics and in the study of the pharmacodynamics of the known non-opioid analgesics and NSAIDs (non-steroidal anti-inflammatory drug). Today, there are the controversial data on the mechanism of a number of non-narcotic analgesics - paracetamol, ketorofen, diclofenac, etc (1-5). Among them, NSAID with the antiviral activity of amizonum can be identified. Amizonum is a strong NSAID: it is more effective than ibuprofen and its' analgesic action corresponds to metamizol (6). The available data do not fully explain analgetic action of amizonum, although it is known that its effect is realized through the reticular formation and peripheral non-opioid mechanisms (7). In the recent years, considerable attention has been paid to the study of the vanilloid component of the analgesic action of new and well-known pain killers. There are data of a vanilloid component mechanism action of the non-opioid analgesic paracetamol (8). We have studied the involvement of vanilloid receptor TRPV1, which plays a significant role in the nociceptive system of the body, and in implementation of the pharmacological action of amizonum.

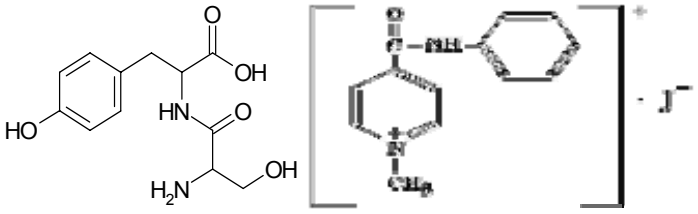
MATERIALS AND METHODS

Animals

In the experiment, male Wistar rats (150-200 g) were used (the animals were bred in the vivarium of the SI "Institute of Pharmacology and Toxicology of NAMS of Ukraine", Kiev, Ukraine). Throughout the experiment the animals were randomised in groups of 5 in cages with the bedding composed of wood shavings (exchanged daily). The animals had free access to a standard commercial diet and water. The animals were kept under a stable regimen of 12 h light/12 h darkness. All studies were performed in accordance with the requirements of the State Expert Center of the Ministry of Health of Ukraine and the rules of the "European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purpose" (Strasbourg city, 1986). Animals were sacrificed by decapitation under ether anesthesia.

Drugs and Analyzers

Amizonum substance (Pharmak pharmaceutical company, Ukraine), agonist TRPV1 capsaicin (Sigma-Aldrich, USA), antagonist TRPV1 capsazepin (Sigma-Aldrich, USA), dipeptide Tyr-Ser (Tyr-Ser), were synthesized in the Department of Synthesis of Biologically Active Substances of the SI "Institute of Pharmacology and Toxicology of NAMS of Ukraine".



AB

Figure 1. *Structural formula of dipeptide Tyr-Ser (A) and amizonum (B)*

Research Methods

The complex formation of amizonum and capsaicin with one of the active sites of TRPV1 by dipeptide Tyr-Ser was studied by Benesi-Hildebrand method (9). The electronic spectra of absorption of capsaicin and amizonum solutions in alcohol were recorded spectrophotometrically. For the purposes of the study, solutions with a constant concentration of these substances were prepared: capsaicin $1 \cdot 10^{-4}$, amizonum $2.5 \cdot 10^{-5}$mol/L and the ratio of substances with Tyr-Ser between 1:2 and 1:32. Stability constant (K_r) was determined using $1/\Delta D$-$1/C_{Tyr\text{-}Ser}$diagram according to the point of intersection with the horizontal axis. Correlation coefficient K_{kor} was calculated. We used the following features: C_B- concentration of alcohol solution Tyr-Ser corresponding to its concentration in the mixture to determine complex formation; C_A/C_B-concentration ratios of A and B substances in the mixture to determine complex formation; D_{kompl}-optical density of the substance to determine complex formation; D_A, D_B-optical density of the substance corresponding to its concentration in the mixture to determine complex formation of A and B, respectively; $D_{\Sigma\,(A+B)}$-the sum of optical densities of A and B substances, in concentrations corresponding to their concentrations in the mixture; $\Delta D = D_{kompl} - D_{\Sigma\,(A+B)}$.

The assessment of the impact of capsaicin, capsazepine, amizonum on TRPV1 in vitro studies carried out in the experiment using the preparation for the portal vein of 4 to 6 mm in length and taken from adult rats was made. The drugs of the vessels were perfused with Krebs solution of the following composition (in mmol/l): NaCl -133; KCl -4.7; $NaHCO_3$ -16.3; $NaHPO_4$ -1.38; $CaCl_2$ -2.5; $MgCl_2$ -1.2; glucose - 7.8; pH -7.4; at the temperature of 37^{0}C. The analgesic was used in aEC_{50} 1,0μmol/L. The substances affecting TRPV1 used in EC_{50}:0.1 μmol/Land 0.5 μmol/L, respectively, for capsaicin and capsazepine (10, 11). The autorhythmic contractile activity of the portal vein is unique physiological feature of this vessel (12). Registration of an isometric force of constriction of the portal vein was performed using a strain gauge attached to the signal amplifier and filter/amplifier. The analog signal was converted to the digital one by the transducer LabTrax (WPI, Inc). Digital data were recorded and written down in the program DataTrax2 (WPI, Inc). Statistical data processing was carried out using the wavelet analysis (13).

The verification in vitro data was carried out on the in vivo model Tail-flick in the another group of animals (14). The test was assessed in groups of 5 rats. The test substances were administered in doses ED_{50}:capsaicin 10 mg/kg s.c.,capsazepine 1 mg/kg i.p., amizonum 25 mg/kg i.m.(15). The tail flick latency was assessed by the analgesiometer (UgoBaile, Italy). The strength of the current passing through the naked nichrome wire was kept constant at 6 Amps. The distance between the heat source and the tail skin was 1.5 cm. The site of application of the radiant heat in the tail was maintained at 2.5 cm, measured from the root of the tail. The cut-off reaction time was fixed at 15 sec to avoid tissue damage. The experiment was conducted according to the scheme: 1) Injection of the agonist/antagonist, then injection of the analgesic (5 minutes later). Measuring was performed 15, 30, 60 min. after the injection of the agonist/ antagonist. 2) Injection of the analgesic, then injection of the agonist/antagonist (5 minutes later). Latency was recorded before and 0.5, 1, 1.5, 2, 2.5 and 3 hours following anadministration of the agents. A static handling of the data was preceded using nonparametric statistics (16). The data were reported as mean ±SD. A level of $P<0.05$ was accepted as statistically significant.

RESULTS

An interaction of capsaicin molecule with Tyr-Ser is characterized with creation of the intermolecular complex at $K_r=0.3\bullet10^4$ l/mol (Figure 1, Table 1).

Spectrophotometric determination of the complex formation of amizonum with Tyr-Ser in alcohol at a wavelength of 246 nm showed that there was the complex formation for which the conditional complexation constant is a small quantity of $K_r=0.05\bullet10^4$ l/mol (Figure 2, Table 2).

The data of the amplitude changes and contractions frequency of the portal vein under the influence of agonist and antagonist of TRPV1, as well as under the influence of amizonum both in mono use and on the background of TRPV1 agonist capsaicin and TRPV1 antagonist capsazepine are shown in Tables 3 and 4. It is shown that both TRPV1 agonist capsaicin and antagonist capsazepine in concentrations 0.1 μmol/L affected the amplitude and frequency of the portal vein contractions, which indicates the presence of vanilloid receptor TRPV1 in this tissue. In this case, the contractions frequency of the portal vein under the influence of capsaicin was reduced by 11.1%-13.2%, and under the influence of capsazepine it increased by 2.0- 4.2%. Under the effect of capsaicin, the amplitude of contractions of the portal vein increased by +30.3%. Capsazepine affects the smooth muscle of the portal vein wall affecting both the amplitude value and the contractions frequency.

Amizonum slightly increased the amplitude of contractions (+7.1%) against the background of a moderate decline in the contractions frequency (Table 3). The applicationof amizonum with capsaicin slightly increases the amplitude of contractions, virtually with no effect on the frequency. The application of amizonum against capsazepine reveals the values of the contractions amplitude and frequency similar to those for capsazepin at mono application.

An injection of amizonum causes a significant analgesic effect in the Tail-flick model, which accounts for a maximum of 30 minutes after administration (Table 5). An injection of amizonum and then capsaicin in animals causes a significant decrease in the latent period of the reaction, up to 44.22% and 54.27% for 15 and 30 min after the start of the experiment, respectively. An injection of capsaicin before amizonum also caused a decrease in the analgesic effect. An injection of capsazepine antagonist TRPV1 before amizonum eliminated its antinociceptive effect. When capsazepine was administered before amizonum, there was a significant antinociceptive effect in comparison with amizonum (Table 5).

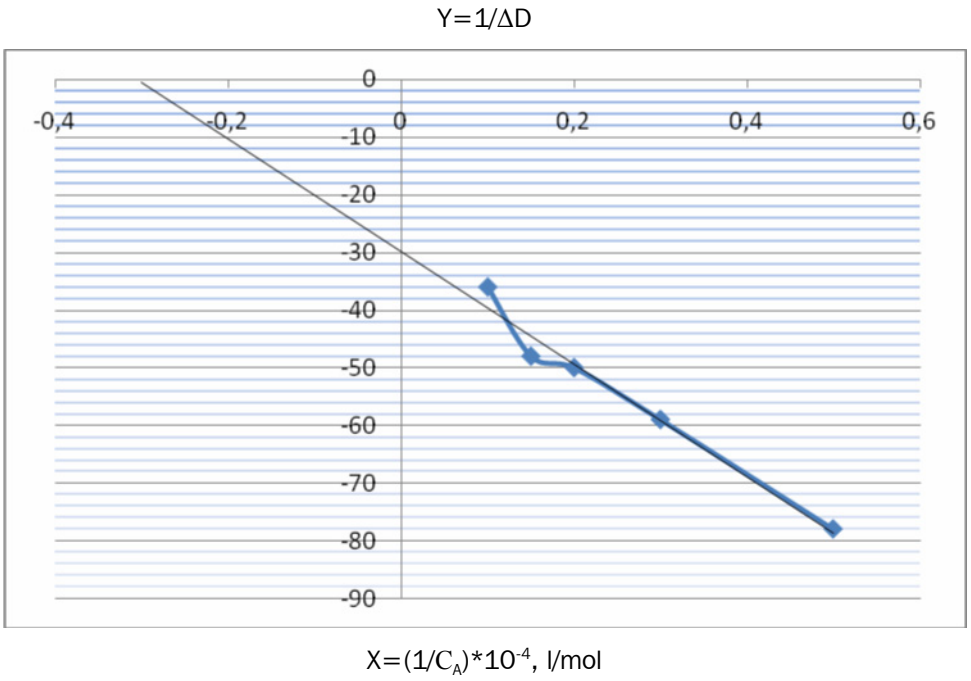


Figure 1. *Spectrophotometric identification of the complex formation of capsaicin (A) with Tyr-Ser(B) in alcohol (concentration of capsaicin is constant $C_A=1\bullet10^{-4}$ mol/l, $\lambda=278$ nm, $D^{\lambda}_{A}=0.263$) Dependence: $1/\Delta D–1/C_B$. $Kr=0.3\bullet10^{4}$ mol/L ($K_{kor}=-0.998$)*

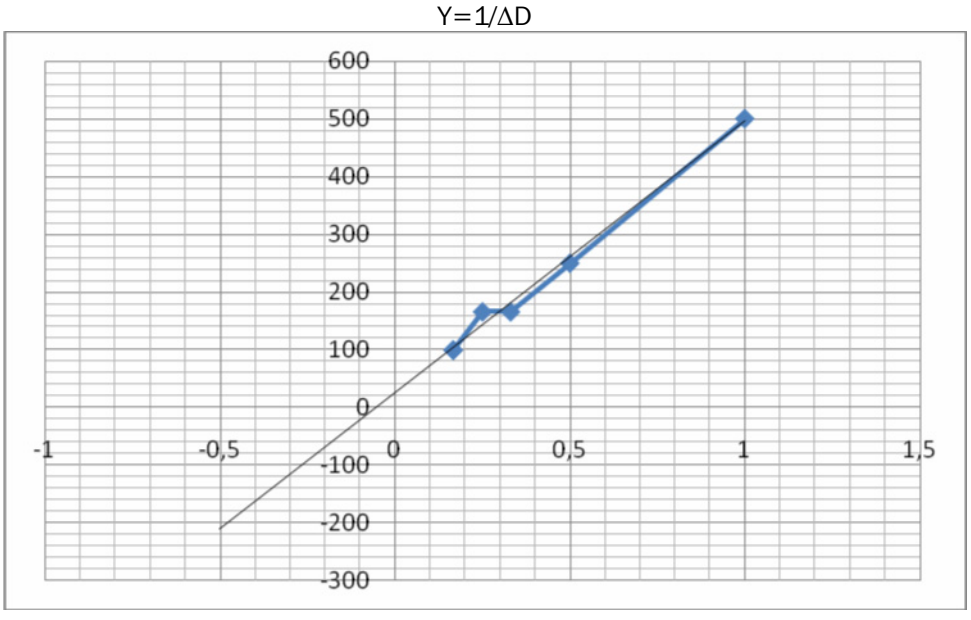


Figure 2. *Spectrophotometric identification of the complex formation of amizonum (A) with Tyr-Ser(B) in alcohol (concentration of amizonum is constant $C_A=1\bullet10^{-4}$ mol/l, $\lambda=246$ nm, $D^{\lambda}_{A}=0.160$). Dependence: $1/\Delta D-1/C_B$. $Kr=0.05\bullet10^{4}$ mol/L ($K_{kor}=-0.995$)*

Table 1. *Spectrophotometric determination of the complex formation of capsaicin (A) with Tyr-Ser(B) in alcohol (concentration of capsaicin is constant $C_A=1\bullet10^{-4}$, mol/L, $\lambda=278$ nm, $D^{\lambda}_{A}=0.263$)*

$C_B \cdot 10^{-4}$mol/L	C_A/C_B	$(1/C_B)\cdot 10^{-4}$mol/l	D_{kompl}	D_A	D_B	$D_{\Sigma(A+B)}$	ΔD	1/ΔD
1	1:2	0.500	0.440	0.263	0.190	0.453	-0.013	- 76.92
2	1:3	0.300	0.529	0.263	0.283	0.546	-0.017	-58.82
3	1:5	0.200	0.715	0.263	0.472	0.735	-0.020	-50.00
4	1:6	0.166	0.812	0.263	0.570	0.833	-0.021	-47.60
5	1:7	0.142	0.910	0.263	0.675	0.938	-0.028	-35.71

Table 2. *Spectrophotometric determination of the complex formation of amizonum (A) with Tyr-Ser(B) in alcohol (concentration of ketorolac is constant $C_A=1\bullet10^{-4}$, mol/L, $\lambda=246$ nm, $D^{\lambda}_{A}=0.160$)*

$C_B \cdot 10^{-4}$mol/L	C_A/C_B	$(1/C_B)\cdot 10^{-4}$mol/l	D_{kompl}	D_A	D_B	$D_{\Sigma(A+B)}$	ΔD	1/ΔD
1	1:1	1.000	0.198	0.160	0.036	0.196	0.002	500.00
2	1:2	0.500	0.236	0.160	0.072	0.232	0.004	250.00
3	1:3	0.333	0.272	0.160	0.106	0.266	0.006	166.67
4	1:4	0.250	0.308	0.160	0.142	0.302	0.006	166.67
5	1:6	0.166	0.388	0.160	0.218	0.378	0.010	100.00

Table 3. *Influence of amizonum, capsaicin and capsazepine on spontaneous activity of the portal vein. The percentages to the norm (in Krebs solution)*

Substance	Amplitude	Frequency
amizonum, n=6	+7.1±3.2%	-8.20±1.37%
capsaicin, n=16	+30.3±5.3%	-11.74±3.70%
capsazepine, n=8	-3.2±2.7%	+6.92±1.93%

The reliability of all studies is 95%

Table 4. *Action of amizonum against application of capsaicin and capsazepine on the spontaneous activity of the portal vein. Percentage changes to the values of activity of capsaicin/capsazepin*

Substance	Amplitude	Frequency
capsaicin+amizonum, n=8	+20.1±1.3%	-7.87±1.90%
capsazepine+amizonum, n=8	-3.00±1.4%	+3.68±1.60%

The reliability of all studies is 95%

***Table 5**. Antinociceptive activity of amizonum after single intramuscular injection against capsaicin and capsazepine on the Tail-flick model, n=5*

Substance	Statistical values	Baseline value	15 min	30 min	60 min
Group			*Latent period of the reaction, sec*		
amizonum	M	4.46	7.88	8.7	5.96
	±m	0.501	0.84	0.85	0.37
changes to the baseline, %	-	-	+76.49**	+94.77*	+33.58
capsaicin	M	4.11	3,31	6,13	5,67
	±m	0.283	0.479	1.527	0.496
changes to the baseline, %	-	-	-19.5	+48.98	+37.65*
amizonum+ capsaicin	M	3.31	4.78	5.117	5.68
	±m	0.28	0.94	0.836	0.70
changes to the baseline, %	-	-	44.22	54.27	71.35**
capsaicin+amizonum	M	3.87	5.47	6.57	6.5
	±m	0.43	0.68	0.88	0.79
changes to the baseline, %	-	-	41.37	69.82*	68.10*
capsazepine	M	4.28	5.78	5.21	5.017
	±m	0.25	0.327	0.36	0.30
changes to the baseline, %	-	-	+35.0*	+21.79	+17.12
capsazepine+amizonum	M	4.83	5.25	4.65	5.96
	±m	0.65	0.62	0.40	0.50
changes to the baseline, %	-	-	+8.6	-4.1	+23.4
amizonum+capsazepine	M	3.85	4.91	5.05	6.23
	±m	0.20	0.277	0.302	0.37
changes to the baseline, %	-	-	+27.5*	+31.1*	+52.2**

Note: *P<0.05 relative to the baseline; **P<0.01 relative to the baseline

DISCUSSION

The structure of capsaicinoids is conditionally divided into three segments: A (4-hydroxy-substituted benzene ring), B (amide or ester fragment), C (aliphatic fragment). It is known that A-segment of capsaicinoids provides up to 75% of the activity, and any modifications of it lead to significant changes in the agonist properties. Capsaicinoids with no B-segment are characterized by smaller affinity to vanilloid receptors and the absence of C- segment results in the reduced activity (17). A- and C-segments are missing in an amizonum molecule, but there is a fragmentary affinity to B-segment (Figure 1). In theory, this group can influence the conformational changes in the structure of vanilloid receptors due to polarization of the radicals of side chains of Ser, Thr, Arg, Lys, His amino acids and depolarization of Asp and Glu radicals under the influence of pH changes in the extracellular space of TRPV1 and others (18). This was the basis to investigate the interaction of amizonum with one of the active sites of vanillod receptor Tyr-Ser. In assessing the binding in the model experiment, the persistent constant (Kr) "Tyr-Ser–amizonum" was considerably lower than the similar value of the complex "Tyr-Ser–capsaicin", which implies a low level of interaction of the analgesic with TRPV1 (9). The further verification of the data in the model experiment was carried out on the portal vein in vitro. In the recent years TRPV1-receptors have been found in the vascular wall of mammals and their physiological role in the body was revealed; some vasoactive peptides (anandamide) affect vascular wall TRPV1 (19, 20). It is shown that their modulation of the functions of cardiovascular system is complex. As such, capsaicin and capsaicin-induced peptides may cause vasodilatation, increased cardiac blood flow, and facilitation of recovery after ischemia (21). On the other hand, there is evidence that 20-hydroxyeicosatrienoic acid (20-

HETE), the arachidonic acid metabolite, causes vasoconstriction by affecting TRPV1 (20). Given these factors, we can talk about the specific reactions of the cardiovascular system under the action of these agents. It was revealed that capsaicin and capsazepine exert the specific effect on the portal veincontractions. Amizonum caused a moderate impact on the spontaneous portal vein contraction (both in the amplitude and in the frequency). Application of this analgesic with capsaicin and with capsazepine revealed moderate changes both in the amplitude and in the frequency of contractions (Table 4). Probably, the membrane mechanisms by which amizonum acts is not affected on spontaneous TRPV1-associated portal vein activity.

The further experimental verification was carried out in vivo experiments in a model of thermal nociceptive stimulation – tail-flick, which characterizes the spinal level of nociception, because spinal neurons contain the largest number of TRPV1 (17). As it is known, under the influence of capsaicin in the body, following TRPV1 excitation, the refractory period occurs, during which the nociceptors are sensitive not only to the action of agonists, but also to the action of other active stimuli (chemical substances, temperature, etc) (17). Therefore, when evaluating the effect of capsaicin on the antinociceptive activity of these substances, we first considered the period up to 30 minutes inclusively after administration, i.e. the period of the prevalence of the analyzer algogenic activity. The group of the animals treated with capsaicin showed decrease of the latent period of the reaction. With injection of capsazepine antagonist TRPV1, in the first 15 minutes, a significant increase in the value of the latent period of the reaction was showed, in comparison with the baseline values with some reduction to 60th minute, which can be explained by the blocking action of the analyzer on the receptor.

Administration of amizonum causes a significant analgesic effect in rats in the model of Tail-flick nociceptive stimulation, which maximizes in 30th minute after the administration (+76.49%). With administration of capsaicin, both before and after amizonum, a marked decline in its antinociceptive activity was shown. The obtained data do not fully correspond to competitive binding with the receptors (22, 23). In a preliminary administration of capsazepine antagonist TRPV1 in the test animals does not show an antinociceptive effect, although both capsazepin and amizonum reveal an analgesic activity in the experiment. When capsazepine was administered after amizonum, there was a significant increase in the latent period of the reaction. However, the antinociceptive effect was much lower than that of amizonum but higher than that of capsazepine.

CONCLUSION

The low value of the constant resistance of the amizonum with Tyr-Ser has failed to reverse the effects of capsaicin and capsazepine in the experiment on the portal vein, as well as the atypical modulation of its effect in the experiment in vivo. This suggests a low probability of TRPV1 involvement in the implementation of the pharmacological effects of amizonum on the organism in the experiment. The data obtained allow us to suppose that the antinociceptive effect of amizonum related to its effect on activation/inhibition of the vanilloid system is not mediated by TRPV1.

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UČEŠĆE VANILOIDNOG RECEPTORA TIPA 1 U MEHANIZMU ANALGETSKOG EFEKTA AMIZONUMA

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Sažetak

Studija karakteristika farmakodinamike novog analgetika je važan i neodložan zadatak savremene farmakologije. Ovakvi podaci omogućavaju razjašnjenje nozologije primene analgetika i stvaranje teorijske pozadine kako bi njegova primena bila optimalna. Efekat posredovan aktivacijom katjonskog kanala tranzitornog receptorskog potencijala, podfamilija V, član 1 (TRPV1), takođe može biti efektivan mehanizam analgetskog dejstva. Procenjivali smo mogućnost učestvovanja TRPV1 u primeni analgetskog efekta uz protiv virusno dejstvo amizonuma u toku eksperimenta. Poznato je da su amino kiseline Tyr511 i Ser512 glavne komponente aktivnog domena TRPV1. U ovoj vezi dipeptid Tyr-Ser se potpuno sintetiše kao model aktivnog domena TRPV1. Ovo je u eksperimentalnom modelu i pokazano primenom spektrofometrijske metode, formiranjem ''kapsaicin-Tyr-Ser'' intermolekularnog kompleksa na nivou konstante stabilnosti K_{kor}=

0.998 i $K_r=0.3\cdot10^{-4}$ L/mol i ''amizonum – Tyr-Ser'' slabog intermolekularnog kompleksa $Kr=0.05\cdot10^{4}$ L/mol; $K_{kor}=0.995$, redom. Provera podataka je u eksperimentu urađena in vitro (izolovana portalna vena pacova) i in vivo (Tail-flick model) pomoću TRPV1 agonista. Pokazano je da je amplituda kontrakcije portalne vene glatkog mišića (GM) pri koncentraciji kapsaicina od 0.1 μmol/L, 0.5 μmol/L capsazepina i 1.0 μmol/L amizonuma bila +30.3±5.3%, -3.2±2.7% and +7.1±3.2% od početnog nivoa, redom. U kombinovanoj primeni amizonuma sa kapsaicinom ili kapsazepinom amplituda kontrakcije portalne vene glatkog mišića bila je 20.1±1.3% i -3.0±1.4%, redom. Ovo ukazuje na odsustvo dejstva amizonuma usled kombinovane primene kapsaicinoida. Tail-flick model je pokazao atipični potencijal antinocicepcije amizonuma upotrebom kapsaicina. Dobijeni podaci ukazuju na malu mogućnost učešća TRPV1 u primeni antinociceptivnog dejstva amizonuma.

Ključne reči: **amizonum, kapsaicin, kapsazepin, dipeptid Tyr-Ser (Tyr-Ser), TRPV1**