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8-[(4-BENZYLPIPERAZIN-1-YL)METHYL]-3-(2-CHLOROPHENYL)-7-HYDROXY-CHROMEN-4-ONE IS AN ACTIVATOR OF CONTRACTILE ACTIVITY OF INTESTINAL SMOOTH MUSCLES WITH REVERSIBLE M2 CHOLINOMIMETIC PROPERTIES

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Background. Several pathologies (such as diabetes mellitus, Parkinson's and Alzheimer's diseases, multiple sclerosis, etc.) are accompanied by degeneration of cholinergic neurons, which are key regulators of the contractile function of the gastrointestinal tract walls, leading to atony and paresis. An effective strategy for normalizing the lack of contractile function of visceral SM is the use of drugs - selective agonists of muscarinic acetylcholine receptors (mAChRs) of the M2 subtype. The high similarity of the structure of the agonist-binding sites of different subtypes of mAChRs causes problems to develop selective ligands for these receptors. Nowadays, there is an urgent necessity to develop selective agonists of M2 subtype receptors as pharmacological tools for laboratory research and promising drugs.

The aim of the present research was to investigate the effect of the 8-[(4-benzylpiperazin-1-yl)methyl]-3-(2-chlorophenyl)-7-hydroxy-chromen-4-one (compound **1**), which was *in silico* predicted to bind mAChRs, on the contractile activity of rat *caecum* circular smooth muscle.

Materials and Methods. The research was carried out on rats. The contractile activity was studied tensometrically in the isometric mode on preparations of the circular smooth muscles of the *caecum* of Wistar rats. The kinetic properties of individual

spontaneous contractions of SM preparations were determined in accordance with the method of multivariate mechanokinetic analysis with the calculation of mechanokinetic parameters for the phases of contraction and relaxation: time (τ_0, τ_C and τ_R), force (F_{max}, F_C and F_R), velocity (V_C and V_R) and impulse (I_{max}, I_C and I_R). The kinetic properties of acetylcholine-induced contractions were analyzed by calculating the normalized maximum velocities of the contraction (V_{nc}) and relaxation (V_{nr}) phases.

Results. It was found that compound **1** caused an increase in the amplitude of acetylcholine-induced contractions; this effect was eliminated by preincubation of SM with the mAChRs M2 subtype inhibitor AF-DX 116.

It was revealed that compound **1** (0.1–50 μM) also has the ability to significantly activate the functional activity of colonic SM in a dose-dependent manner, increasing the force and frequency of spontaneous contractions, as well as their mechanokinetic parameters.

It was found that the presence of compound **1** (0.1 μM) in the solution washing the smooth muscle for a long time leads to a significant increase in the amplitude and frequency of spontaneous contractions, which tends to reach a stationary mode after 40 minutes of its action. The effect of compound **1** was stable for at least an hour of application to the *caecum*, and was reversible and significantly eliminated by washing the SM.

Conclusions. Compound **1** stimulates the contractile activity of the cecal smooth muscle and exhibits M2 cholinergic properties.

Keywords: smooth muscle of the large intestine, muscarinic acetylcholine receptors of the M2 subtype, acetylcholine, spontaneous contractions, mechanokinetic analysis

INTRODUCTION

One of the macrostructural components of the gastrointestinal tract (GIT) is smooth muscle tissue, which is located in several layers, such as the outer longitudinal and inner circular layers (Al-Shboul, 2013; Kola *et al.*, 2022; López-Pingarrón *et al.*, 2023). Between the smooth muscle layers and the submucosa, there are two layers of neural cells of the myoenteral and submucosal nerve plexuses (enteric nervous system) (Furness *et al.*, 2014; Veress *et al.*, 2022; Windster *et al.*, 2023). In addition to „working" contractile cells and neurons, the gastrointestinal smooth muscle tissue contains interstitial cells of Cajal (ICC) that function as pacemakers. Importantly, the pacemaker properties of ICCs are regulated by the release of neurotransmitters from enteric neurons.

In gastrointestinal smooth muscle (SM) tissue, acetylcholine is the main excitatory neurotransmitter that mediates the activation of receptors coupled to heterotrimeric G-proteins and provides for the activation of contractile activity (Zhang *et al.*, 2011; Balla *et al.*, 2023). These receptors, called muscarinic acetylcholine receptors (mAChRs), are represented in the stomach and intestinal SM cells by subtypes M2 (dominant, with a stoichiometry of 75–80 %), M3 (about 20–25 %), and M1 (probably expressed in minimal amounts) (Tobin *et al.*, 2009; Tanahashi *et al.*, 2020, 2021). Receptors of the M3 subtype (like M1) are coupled to G-proteins belonging to the $G_{q/11}$ class. Therefore, their activation mainly causes stimulation of phospholipase C_β with subsequent synthesis of secondary messengers inositol 1,4,5-triphosphate (IP_3) and diacylglycerol. Additionally, an increase in the inward cationic current through TRPC4 and TRPC6 channels is a factor in muscle cell excitation when these receptors are stimulated (Tsvilovskyy *et al.*, 2009;

Zholos *et al.*, 2023). M2 subtype receptors are coupled to $G_{i/o}$ proteins, and their activation causes contraction of the SM by several mechanisms: inhibition of adenylate cyclase activity and reduction of cAMP concentration, as well as activation of the inward cationic current through TRPC4 channel (Zholos, 2006; Tanahashi *et al.*, 2021).

Several pathologies (such as diabetes mellitus, Parkinson's and Alzheimer's diseases, multiple sclerosis, etc.) are accompanied by degeneration of neurons that regulate the contractile function of the gastrointestinal walls, leading to atony and paresis (Glick *et al.*, 1982; Semar *et al.*, 2013; Puig *et al.*, 2015; Thomzig *et al.*, 2021; Liu *et al.*, 2022; Yang *et al.*, 2023). In particular, in Parkinson's disease, which is accompanied by hypofunctional motor activity of the SM of the gastrointestinal tract in 80 % of patients, α-synuclein-mediated destruction of cholinergic neurons of the vagus nerve (Liu *et al.*, 2022) and in the enteric nerve plexuses (Ma *et al.*, 2019; Fricova *et al.*, 2020) is observed. In the case of Alzheimer's disease pathogenesis, as shown in in vitro model studies, extracellular tau protein binds directly to mAChRs of M1 and M3 subtypes and causes an increase in the intracellular concentration of Ca^{2+} ions, which may be one of the important causes of neuronal death (Wysocka *et al.*, 2020; Jimenez *et al.*, 2023).

The high similarity of the agonist-binding sites of mAChRs of different subtypes (Pegoli *et al.*, 2019) as well as the co-expression of several receptor subtypes in the same tissues cause problems with the development of selective ligands for these receptors (Nyporko *et al.*, 2023). Hence, currently, substances that have only relative selectivity for certain receptor subtypes are used as active ingredients in medicines. Such a relatively selective agonist for the M2 subtype of mAChRs (compared to the M4 subtype) is carbamyl-β-methylcholine (EC_{50} for M2 receptors is 25 μM, while for the M4 subtype it is 317 μM). In the case of M3 receptors, relatively selective agonists are zamifenacin, darifenacin and pF-HHSiD (Caulfield & Birdsall, 1998; Greig *et al.*, 2013). Thus, the necessity of developing selective M2 receptor agonists as pharmacological tools for laboratory research and promising drugs is extremely urgent. In contrast, a significant amount of data has been accumulated to date indicating a direct interrelation between increased activation and hyperactivation of M3 subtype mAChRs by pharmacological agents and the development of tumours, particularly in the gastrointestinal tract (Felton *et al.*, 2018; Hering *et al.*, 2021; Lobbes *et al.*, 2023). Therefore, the most successful strategy to normalise the insufficient contractile function of visceral SMs is the use of drugs - selective agonists of mAChRs of the M2 subtype.

The rational design of pharmacologically active compounds using several methods of computer structural biological analysis and modelling, receptor-oriented virtual screening and modern combinatorial chemistry is the most successful and progressive strategy to create compounds with selective action on individual cellular targets (Nyporko *et al.*, 2023). The aim of this study is to investigate the effect of compound 8-[(4-benzylpiperazin-1-yl)methyl]-3-(2-chlorophenyl)-7-hydroxy-chromen-4-one (compound **1**), for which the ability to activate mAChRs was predicted by *in silico* methods on the contractile activity of rat *caecum* circular SMs.

MATERIALS AND METHODS

Tensometric experiments. The studies were performed on Wistar rats of the vivarium population of the State Institution "Institute of Pharmacology and Toxicology of the National Academy of Medical Sciences of Ukraine". The rats were maintained on a standard diet and under standard conditions: temperature 20±2 °C, relative humidity

50–70 %, light conditions – light:dark = 12:12 hours. Rats were euthanized by dislocation of the cervical vertebrae under chloroform anaesthesia. All animal manipulations were carried out in accordance with the International Convention for the Protection of Animals and the Law of Ukraine "On the Protection of Animals from Cruelty" (the Minutes of the meeting of bioethics commission of SSC Institute of Biology and Medicine No. 3 dated May 2, 2019).

The contractile activity was studied tensometrically in the isometric mode on preparations of the circular *caecum* SM. The *caecum* muscles were preliminarily cleaned of mucosa, cut in a circular direction into preparations (average size 1.2×7 mm) and placed in a working chamber (volume 2 mL) with a flowing Krebs solution (flow rate – 5 mL/min), thermostated at 37 °C; preparations were subjected to passive tension (10 mN) and left for 1 hour.

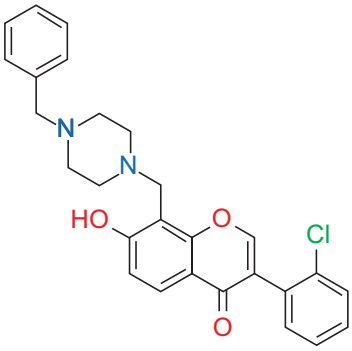

Fig. 1. Structure of the compound **1**

Krebs solution (mM) was used for the experiments: 120.4 NaCl; 5.9 KCl; 15.5 $NaHCO_3$; 1.2 NaH_2PO_4; 1.2 $MgCl_2$; 2.5 $CaCl_2$; 11.5 glucose; pH 7.4. Acetylcholine (10 μM) and the M2 subtype receptor antagonist compound AF-DX 116 (10 μM) (both manufactured by Sigma) were used in the study.

Compound **1** (8-[(4-benzylpiperazin-1-yl)methyl]-3-(2-chlorophenyl)-7-hydroxy-chromen-4-one) (**Fig. 1**) was provided by a supplier of HTS compounds OTAVA Ltd (Kyiv, Ukraine).

When the compound was used, a stock solution in dimethyl sulfoxide (DMSO) was preliminarily prepared at the rate of 0.25 % of the substance in the final volume. Also, all control studies of the contractile activity of *caecum* were carried out under the action of 0.25 % DMSO.

Mechanokinetic analysis. The kinetic properties of individual spontaneous contractions of SM preparations were determined according to the method of multiparametric mechanokinetic analysis (Kosterin *et al.*, 2021). The analysis of the complete profile of single spontaneous contractions was based on the procedure of their linearisation in the coordinates $\left[\ln\left(\frac{f_R}{f_C}\right);\ln\left(1+\Delta t/t\right)\right]$ (where f and t are instantaneous values of force and time, and the indeces C and R denote the phases of contraction and relaxation, respectively). Hereafter, F_C and F_R are, respectively, the force values at the inflection points of the mechanogram at the level of the contraction (from the beginning of the force increase to its amplitude value F_{max} at time τ_0) and relaxation (from the amplitude value F_{max} to the basal tension level) phases. The value of time interval Δt was chosen arbitrarily.

The characteristic constants k and n were determined from the linearized graphs, on the basis of which the mechanokinetic parameters of the contraction and relaxation phases were calculated: time (τ_0, τ_C and τ_R), force (F_{max}, F_C and F_R), velocity (V_C and V_R – maximum velocities of the contraction and relaxation phases, respectively) and impulse (I_{max}, I_C and I_R – parameters of the force impulse at the level of amplitude and maximum velocities of the contraction and relaxation phases, respectively).

Analysis of the kinetic properties of acetylcholine-evoked contractions was carried out according to the method described earlier (Burdyga & Kosterin, 1991). In the process of analysis, parameters independent of the amplitude of contractile responses were calculated: normalized maximum velocities of the contraction (V_{nc}) and relaxation (V_{nr}) phases.

Statistical analysis. The experimental data were processed by methods of variation statistics using the Origin 2018 software. The samples were tested for their belonging to normally distributed statistical populations using the Shapiro-Wilk's test. The one-way analysis of variance (ANOVA) was used to determine significant differences between the sample means. The results were considered significant if the probability value was $p < 0.05$. The reliability of the data approximation during mechanokinetic analysis by a linear function was analysed using the Fisher's test; in all cases, the coefficients of determination (R^2) were ≥0.96. The results are expressed as mean ± standard error of the mean, n – number of experiments.

RESULTS AND DISCUSSION

Modulation of *caecum* acetylcholine-induced contractions by compound 1. To determine the effects of compound **1** on acetylcholine (10 μM) induced contractions, muscle preparations were pre-incubated with the compound (1 μM) for 5 min. It should be noted that the addition of compound **1** to the solution washing the smooth muscle preparations at a concentration of 1 μM did not cause a phase contraction, but under these conditions, an increase in spontaneous *caecum* contractions was observed, so this effect was investigated in detail further.

The addition of acetylcholine in combination with compound **1** caused an increase in the amplitude of acetylcholine-induced contractions to an average of 134.6±4.1 % relative to the control taken as 100 % ($p < 0.01$, n = 5) (**Fig. 2**). It is also noteworthy that the tonic component of acetylcholine-induced contractions increased by a similar amount (on average to 134.1±3.9 %, $p < 0.01$, n = 5).

The mechanokinetic analysis revealed that compound **1** modulated the relaxation phase, providing a significantly more efficient contractile response. Thus, the normalised maximum velocity of the contraction phase (V_{nc}) did not differ significantly from the control and averaged 108.8 ± 7.1 % ($p > 0.05$, n = 5), while the corresponding relaxation phase (V_{nr}) significantly decreased on average to 51.7±4.3 % ($p < 0.001$, n = 5).

The increase in the amplitude of the acetylcholine-evoked contraction accompanied by an increase in the tonic component and a selective slowing of the normalised rate of the relaxation phase under the influence of compound **1** suggests the ability of this substance to activate M2 subtype mAChRs (Elorriaga *et al.*, 1996; Wrzos *et al.*, 1996; Tanahashi *et al.*, 2021).

To test the hypothesis regarding the effect of compound **1** specifically on M2 subtype mAChRs, acetylcholine-induced contractions of SM *caecum* were recorded under the conditions of prior inhibition of receptors of this subtype with the substance 11-[[2-[(Diethylamino)methyl]-1-piperidinyl]acetyl]-5,1 1-dihydro-6H-pyrido[2,3-b][1,4] benzodiazepin-6-one (code AF-DX 116), which is an competitive inhibitor of mAChRs with greater efficiency against M2 (IC50 0.36 μM) and M4 (IC50 0.79 μM) receptor subtypes (Buckley *et al.*, 1988). It was established that the use of AF-DX 116 (10 μM, duration of preincubation 20 min) caused a decrease in the amplitude of acetylcholine-induced contractions of the SM (on average by 21.4±5.1 %, $p < 0.05$, n = 5). Under the

influence of AF-DX 116, the application of compound **1** (1 μM) did not cause activation of acetylcholine contractions of the *caecum*, and their amplitude was on average 78.9±8.3 % compared to the action of AF-DX 116 ($p > 0.05$, $n = 5$). Thus, the obtained results confirm the predictions regarding the mechanism of action of compound **1** as an agonist of mAChRs M2 subtype.

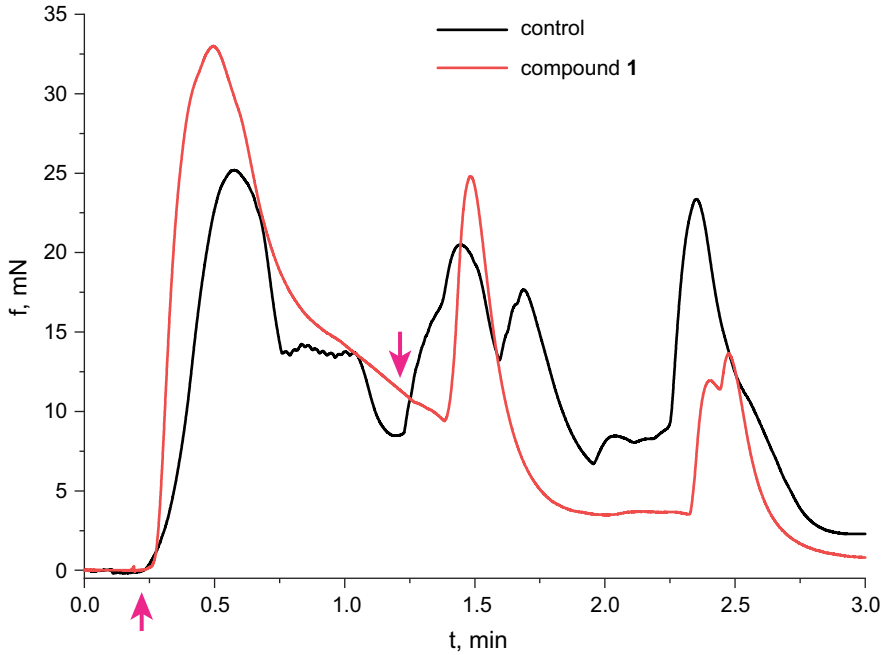

Fig. 2. Typical mechanograms of acetylcholine (1 μM) induced contractions of rat *caecum* circular smooth muscle in control and after pre-incubation for 5 min with 1 μM of compound **1**. The moment of addition of acetylcholine is shown by the arrow

Dose effects of compound 1 on rat *caecum* functioning. In the next stage, we investigated the mechanokinetic effects of a cumulative application of compound **1** in concentrations of 0.1, 1, 10 and 50 μM to the SM *caecum*, with each concentration lasting for 10 min.

The application of compound **1** at a minimum concentration of 0.1 μM did not cause a phase contraction of *caecum* preparations, but activated their spontaneous contractile activity (**Fig. 3**). Thus, under these conditions, a significant increase in the frequency of spontaneous contractions was observed (on average to 168.8±9.2 %, $p < 0.01$, $n = 5$); there was also a tendency to increase the amplitude of spontaneous contractions (129.7±8.6 %, $p = 0.079$, $n = 5$).

A cumulative increase in the concentration of compound **1** to 1 μM caused further activation of the contractile activity of *caecum* preparations: under these conditions, the frequency of spontaneous contractions averaged 572.6 ± 23.4 % ($p < 0.001$, $n = 5$) and their amplitude was 281.3±11.7 % ($p < 0.001$, $n=5$) relative to the corresponding control parameters.

A further increase in the concentration of compound **1** to 10 μM was accompanied by a continued activation of the spontaneous contractile activity of SM *caecum*. These

changes can be mainly attributed to the amplitude (mean 508.8±27.9 % relative to the control, n = 5), since it was significantly different both relative to the control ($p < 0.001$) and to the data of exposure to compound **1** at a concentration of 1 μM ($p < 0.001$) (**Fig. 4*A***). The frequency of spontaneous contractions under these conditions was significantly different from the control value (614.6±35.1 % relative to the control, n = 5, $p < 0.001$), but not from the frequency under the effect of the compound at a concentration of 1 μM ($p > 0.05$).

The use of the next concentration of compound **1** (50 μM) caused a continued increase in the activation of spontaneous contractions of SM preparations: under these conditions, their amplitude averaged 605.6±31.3 % relative to the control ($p < 0.001$, n = 5), which was also significantly higher than under the action of 10 μM ($p < 0.01$) (**Fig. 4*A***). Furthermore, the frequency of spontaneous contractions increased significantly (on average to 738.1±44.6 % relative to the control, n = 5, $p < 0.001$).

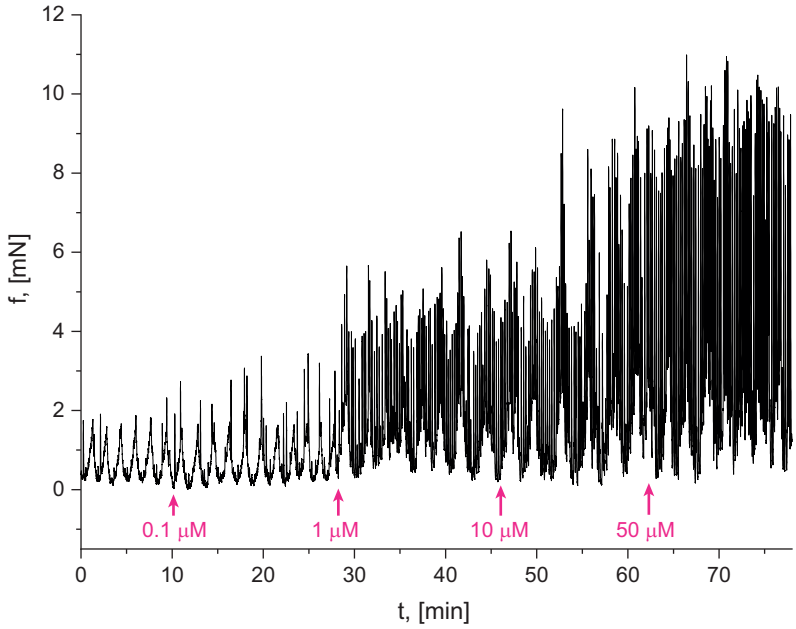

Fig. 3. Spontaneous contractile activity of rat *caecum* circular smooth muscles in control and after cumulative addition of compound **1** to the washing solution at concentrations of 0.1–50 μM, as well as its washing. A typical mechanogram is shown. Arrows indicate the moments of addition of compound **1** in the indicated concentrations

To obtain additional information about the effects of compound **1** on the functioning of rat *caecum* SM, individual spontaneous contractions were analysed using multivariate mechanokinetic analysis with the calculation of time (τ_0, τ_C and τ_R), force (F_C and F_R), velocity (V_C and V_R) and impulse (I_{max}, I_C and I_R) parameters (Kosterin *et al.*, 2021) (**Fig. 4**).

It was found that compound **1** in all used concentrations (0.1–50 μM) caused a significant dose-dependent increase in the maximum force parameter at the level of the relaxation phase (F_R). The maximum force parameter of the contraction phase (F_C) demonstrated a dose-dependent increase under the cumulative addition of compound **1** at higher concentrations (1–50 μM) (**Fig. 4*A***). Compound **1** did not affect the time parameters of spontaneous contractions (**Fig. 4*B***).

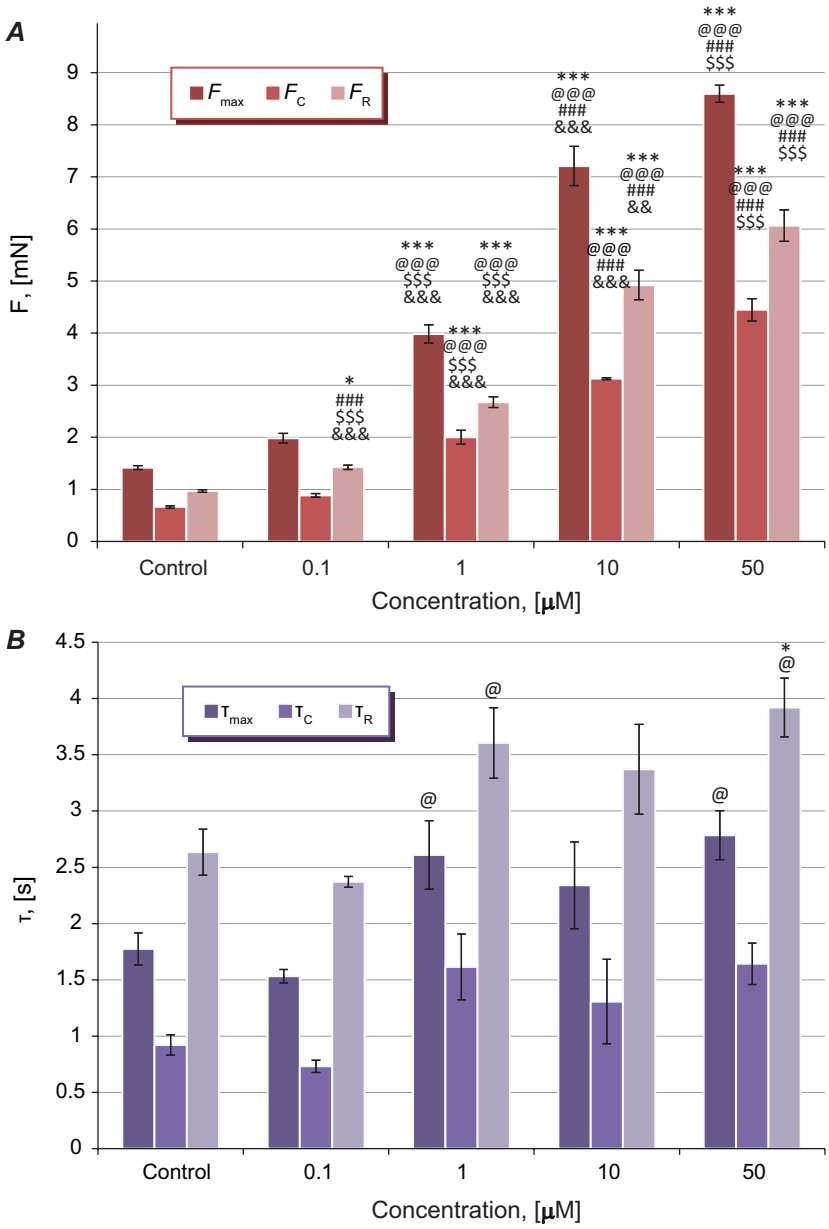

Fig. 4. Mechanokinetic parameters of spontaneous contractions of rat *caecum* circular smooth muscles in control and after cumulative addition of compound **1** to the washing solution at concentrations of 0.1–50 μM (n = 5, M ± m): ***A*** – force parameters (F_{max}, F_C and F_R); ***B*** – time parameters (τ_0, τ_C and τ_R). * – p <0.05 and ** – p <0.01 – relative to the control; statistically significant for different concentrations of compound **1**: @ – p <0.05 and @@@ – p <0.001 – for 0.1 μM; ### – p <0.001 – for 1 μM; $$$ – p <0.001 – for 10 μM; && – p <0.01 and &&& – p <0.001 – for 50 μM

Normalization of both F_C and F_R to the amplitude values (F_{max}) completely eliminated the effects of compound **1**, which allows us to predict the general activation of

the processes of increasing the concentration of Ca^{2+} ions in the myoplasm of SM cells under the influence of compound **1**, but the absence of a specific effect of this compound on the processes of active extrusion of these cations (Burdyga & Wray, 2002; Tsymbalyuk *et al.*, 2016, 2019, 2021).

Additionally, in the presence of compound **1** in higher concentrations (1–50 μM), an increase in the absolute values of the maximum velocities of the phases of contraction (V_C) and relaxation (V_R) was observed (**Fig. 5*A***). In the case of velocity parameters, the dose dependence was weakly expressed, and the activation effects in the background of 10 and 50 μM compound **1** did not differ. However, it is worth noting that the V_C/V_R ratio in all cases of compound **1** exposure did not differ significantly from the control, which also suggests the absence of effects of this substance on the processes of energy-dependent Ca^{2+} transport (Tsymbalyuk *et al.*, 2016, 2019).

The impulse parameters (I_{max}, I_C and I_R) were equally significantly and dose-dependently changed in the presence of 1–50 μM of compound **1**, reaching a tenfold increase in the case of the highest concentration used (**Fig. 5*B***). Such an increase in force impulse under the influence of compound **1** cannot be explained only by an increase in contraction force, because normalization of impulse parameters to F_{max} still indicates a significant, on average, one and a half times increase.

Thus, the results of the study indicate that compound ***1*** has the ability to significantly activate the functional activity of colonic SMs in a dose-dependent manner, increasing the force of spontaneous contractions, probably by increasing the concentration of Ca^{2+} ions in the myocyte cytoplasm during their excitation (Elorriaga *et al.*, 1996; Wrzos *et al.*, 2004). A significant (more than seven-fold in the presence of 50 μM of the compound) increase in the frequency of spontaneous contractions in the presence of compound **1** indicates activation of the pacemaker activity of the ICC (Fujimoto *et al.*, 2004; Sanders, 2019). At the same time, even with high concentrations of **1**, we did not record phasic contractions, as in the case of acetylcholine (**Fig. 2**). Thus, this substance is not a non-selective full agonist of acetylcholine receptors, but may be either a partial agonist or an agonist of the mAChRs M2 subtype. The selective action of compound **1** is supported by the results of studies of the functional activity of the SM of gastrointestinal tract of mice with knockout of M3- and M2-acetylcholine receptors. In the case of the presence of the M2 subtype of mAChRs alone, acetylcholine application caused a significant increase in the spontaneous contractile activity of the intestine SM accompanied by very weakly expressed phase-controlled contractions (Unno *et al.*, 2005).

Investigation of the time-dependent effects of compound 1 on the functions of the rat *caecum* and its washout efficiency. During drug development, it is crucial that the effects of their active substances remain stable over time, avoiding the risk of efficacy loss or the emergence of opposite effects when applied to the target tissue for an extended duration. Therefore, in the next step, we investigated the time effects of the application of 0.1 μM of compound **1** for an hour on the mechanokinetic parameters of spontaneous contractile activity of SM *caecum* preparations.

As can be seen from **Fig. 6**, the application of compound **1** for a long time leads to a significant increase in spontaneous contractile activity, which tends to reach a stationary mode after 40 min of its action. Thus, after 30 min of exposure to compound **1**, a significant increase in the amplitude of spontaneous contractions (F_{max}) was observed not only relative to the control ($p < 0.001$), but also relative to the effects after 10 min

of exposure to this compound (p <0.05). After application of this compound for 1 hour, the amplitude further increased and averaged 242.3±11.8 % (p <0.001 for control and 10 min of exposure, and p<0.01 for 30 min of exposure, n = 5) (**Fig. 7*A***).

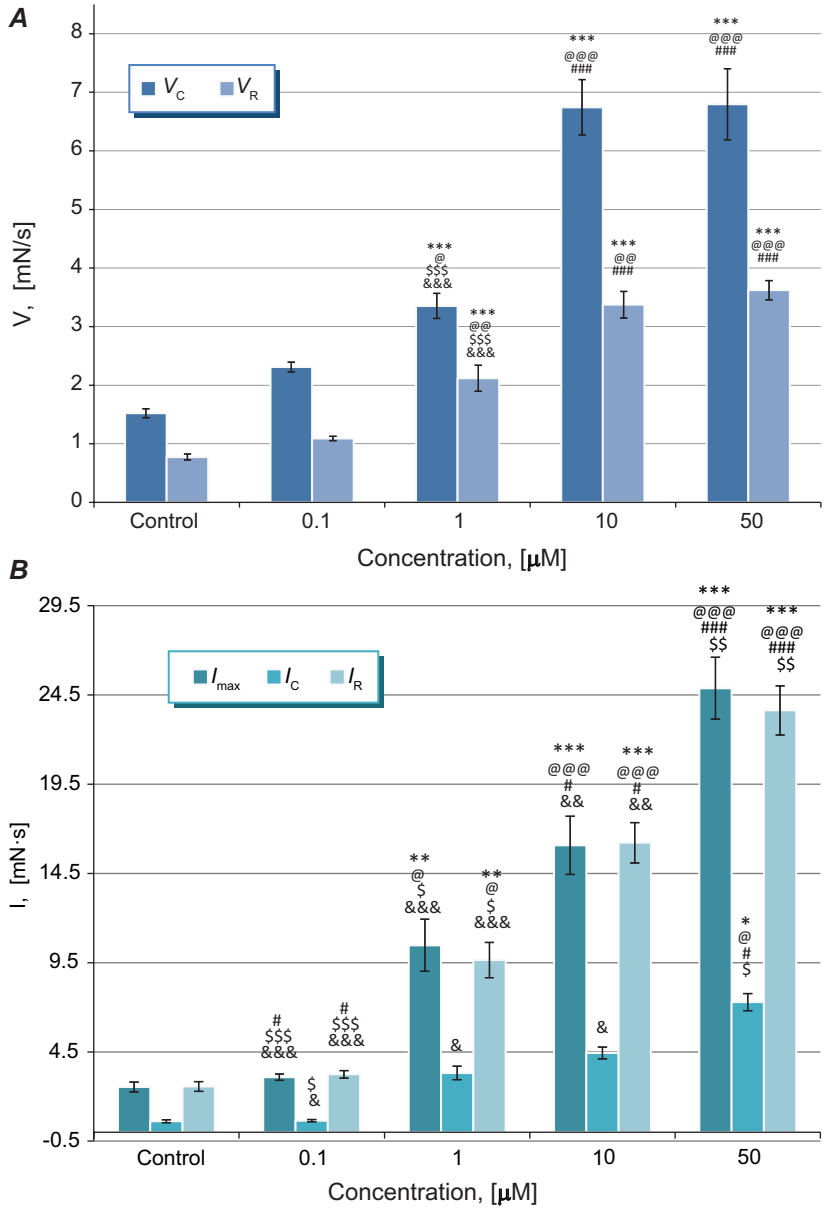

Fig. 5. Mechanokinetic parameters of spontaneous contractions of rat *caecum* circular smooth muscles in control and after a cumulative addition of compound **1** to the washing solution at concentrations of 0.1–50 μM (n = 5, M ± m): ***A*** – velocity parameters (V_C and V_R); ***B*** – impulse parameters (I_{max}, I_C and I_R). * – p <0.05, ** – p <0.01 and *** – p <0.001 – relative to the control; statistically significant for different concentrations of compound **1**: @ – p <0.05, @@ – p <0.01, @@@ – p <0.001 – relative to 0.1 μM; # – p <0.05, ## – p <0.01, ### – p <0.001 – relative to 1 μM; $ – p <0. 05, $$ – p <0.01, $$$ – p <0.001 – relative to 10 μM; & – p <0.05, && – p <0.01 and &&& – p <0.001 – relative to 50 μM

It is pertinent to mention that as the duration of incubation of SM preparations with compound **1** increased, the frequency of spontaneous contractions also increased in a direct proportion, which suggests that under these conditions the pacemaker activity of the ICC was stimulated (Fujimoto *et al.*, 2004; Sanders, 2019).

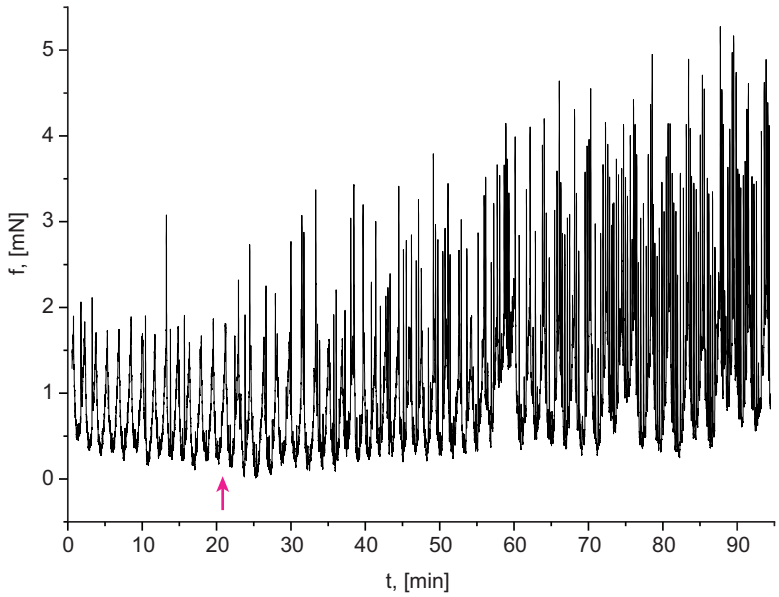

Fig. 6. Spontaneous contractile activity of rat *caecum* circular smooth muscle in control and after application of compound **1** at a concentration of 0.1 μM. A typical mechanogram is shown. The arrow indicates the moment of addition of compound **1**

To determine the quantitative patterns of activation of *caecum* SM motor activity at different durations of exposure to compound **1**, individual cycles of spontaneous contractions were examined according to the method of multiparameter analysis (Kosterin *et al.*, 2021). It was found that under these conditions, there was a significant time-dependent increase in force parameters (F_{max}, F_C and F_R) (**Fig. 7*A***). However, under different durations of compound **1** application, as well as in the case of exposure to different concentrations of this compound **(Fig. 4*B***), the time parameters of spontaneous contractions did not undergo significant changes (**Fig. 7*B***).

In addition, under different durations of treatment with compound **1**, a significant time-dependent increase in both velocity parameters of spontaneous contractile activity of *caecum* SM was observed only with its prolonged application (**Fig. 8*A***). Thus, the maximum velocity of the contraction phase was significantly different only in comparison with the control group, while the velocity of the relaxation phase was also different in comparison with the application of the compound for 10 and 30 min. In the case of impulse parameters (I_{max}, I_C and I_R), a significant time-dependent increase was observed when **1** was applied to SM preparations for 30 and 60 min (**Fig. 8*B***). Therefore, it is important to note that compound **1** does not lose its efficiency during prolonged in vitro application.

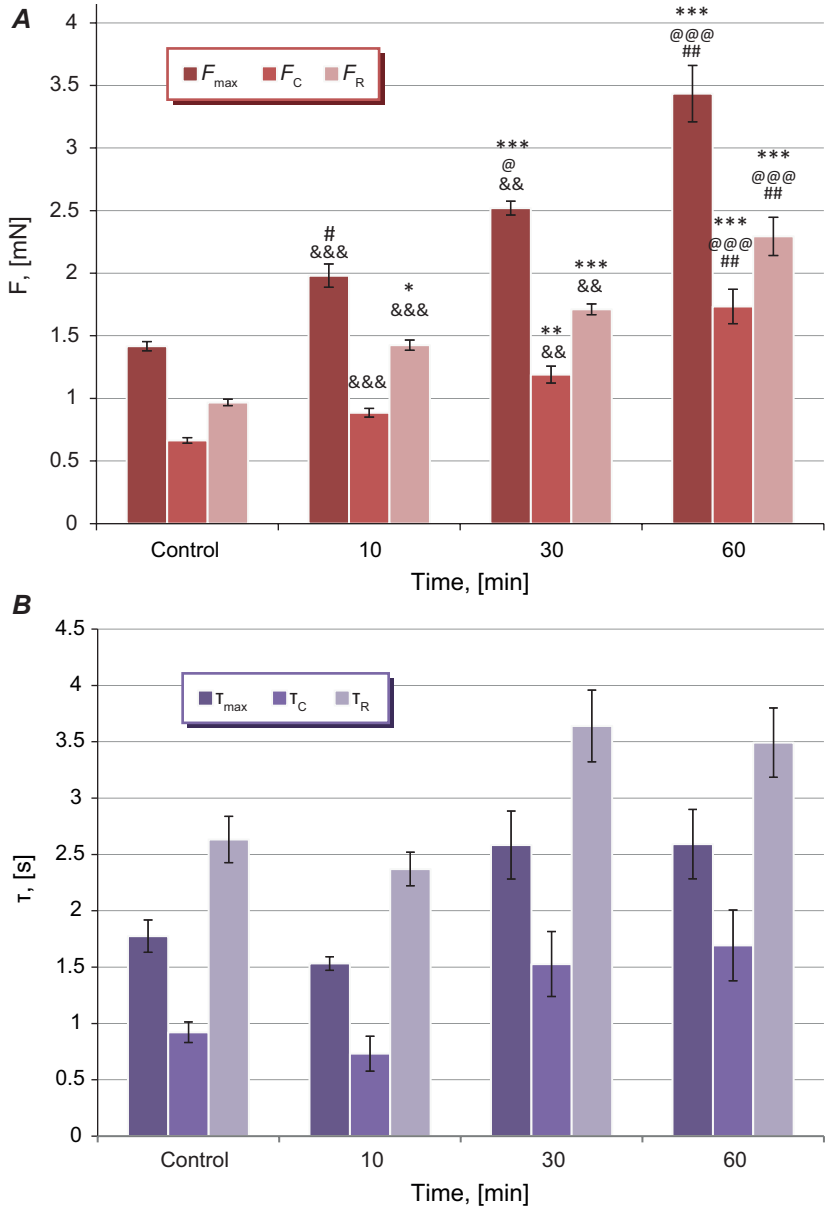

Fig. 7. Mechanokinetic parameters of spontaneous contractions of rat *caecum* circular smooth muscle in control and under the influence of compound **1** (0.1 μM) for 10, 30 and 60 min (n = 5, M ± m): ***A*** – force parameters (F_{max}, F_C and F_R); ***B*** – time parameters (τ_0, τ_C and τ_R).
* – p <0.05, ** – p <0.01 and *** – p <0.001 – relative to the control; statistically significant for different durations of exposure to compound **1**: @ – p <0.05 and @@@ – p <0.001 – for 10 min; # – p <0.05, ## – p <0.01 and ### – p <0.001 – for 30 min; & – p <0.05, && – p <0.01 and &&& – p <0.001 – for 60 min

Another important property of substances that can be used as a basis for drug development is the reversibility of their action. Therefore, we further tested the ability to wash out compound **1** by replacing the Krebs solution containing 50 μM of the substance

used to wash SM drugs with normal Krebs solution. As can be seen from **Fig. 9*A***, the replacement of the modified solution with normal Krebs solution was accompanied by a decrease in the amplitude of spontaneous contractions; however, their frequency remained unchanged.

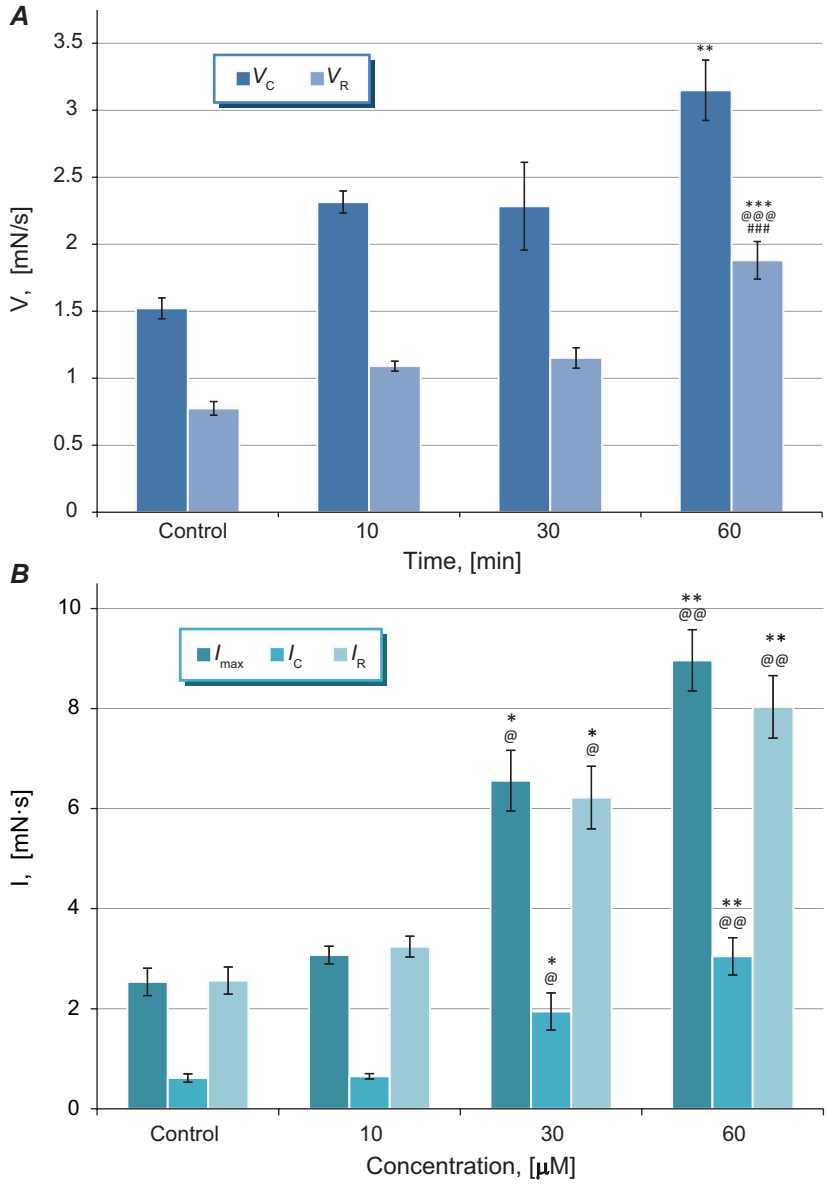

Fig. 8. Mechanokinetic parameters of spontaneous contractions of rat *caecum* circular smooth muscles in control and under the influence of compound **1** (0.1 μM) for 10, 30 and 60 min (n = 5, M ± m): ***A*** – velocity parameters (V_C and V_R); ***B*** – impulse parameters (I_{max}, I_C and I_R).
* – p <0.05, ** – p <0.01 and *** – p <0.001 – relative to the control; statistically significant for different concentrations of compound **1**: @ – p <0.05, @@ – p <0.01, @@@ – p <0.001 – relative to 0.1 μM; # – p <0.05, ## – p <0.01, ### – p <0.001 – relative to 1 μM; $ – p <0. 05, $$ – p <0.01, $$$ – p <0.001 – relative to 10 μM; & – p <0.05, && – p <0.01 and &&& – p <0.001 – relative to 50 μM

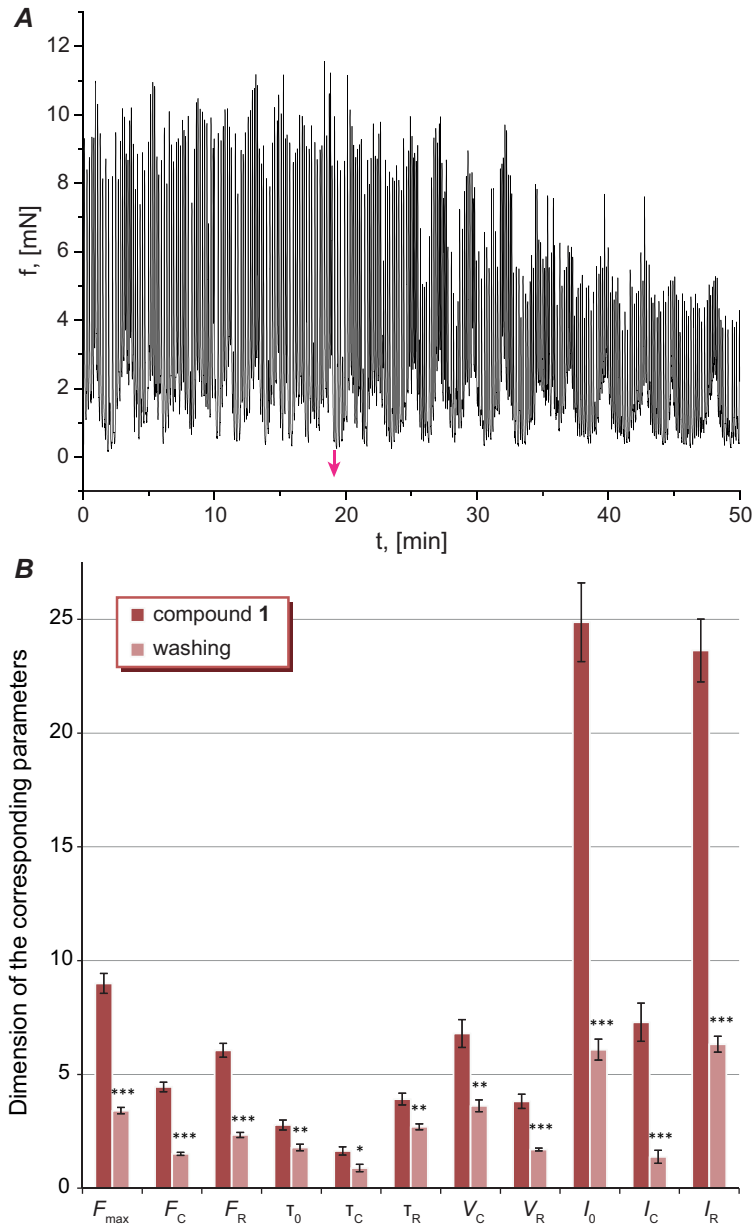

Fig. 9. Spontaneous contractile activity of rat *caecum* circular smooth muscles under the influence of compound **1** (50 μM) and after its washing with normal Krebs solution (the moment of the washing start is shown by an arrow); a typical mechanogram is shown (***A***). ***B***, mechanokinetic parameters of individual spontaneous contractions in the presence of compound **1** (50 μM) and after its washing (n = 5, M ± m): force parameters (F_{max}, F_C and F_R; their dimension [mN]); time parameters (τ_0, τ_C and τ_R; their dimension [s]); velocity parameters (V_C and V_R; their dimension [mN/s]); impulse parameters (I_{max}, I_C and I_R; their dimension [mN*s]). The arrow shows the moment of the beginning of the washout of compound **1**.
* – p <0.05, ** – p <0.01 and *** – p <0.001 – for the effect of compound **1** (50 μM)

Subsequently, a fragment of the mechanogram was evaluated using mechanokinetic analysis 20 min after the beginning of drug washing. It was found that during washing all groups of parameters, without exception, significantly changed (**Fig. 9*B***). Even though, during this time only the time parameters (τ_0, τ_C and τ_R) and the force pulse during the contraction phase (I_C) reached the control level, the obtained data indicate the reversibility of the action of compound **1**.

In gastrointestinal smooth muscle tissue, mAChRs are expressed in “working” myocytes and ICCs that play a fundamental role in the perception of the main excitatory neurotransmitter acetylcholine. Although subtype M2 receptors are dominant in both cell types, the direct response of mAChRs to acetylcholine activation by M2 subtype is weakly expressed (Iino & Nojyo, 2006; Ehlert, 2003). *In vivo*, their functional role is to promote depolarisation of the plasma membrane of cells by indirectly activating the inward cationic current through TRPC4 channels and L-type Ca^{2+} channels (Unno *et al.*, 2006; Zholos, 2006), as well as inhibition of adenylate cyclase activity and reduction of cAMP concentration, counteracting the effects of inhibitory neurotransmitters, in particular, norepinephrine (Ehlert, 2003; Balla *et al.*, 2023). Therefore, selective activation of the M2 subtype of mAChRs by selective agonists could be useful to normalise many pathologies accompanied by impaired visceral smooth muscle motility (Kim *et al.*, 2019; Yan *et al.*, 2021).

Compound **1**, which was predicted to activate mAChRs by *in silico* methods, *in vitro* showed the ability to activate acetylcholine-induced contractions and to enhance spontaneous motility of colon SM in a dose-dependent manner, increasing their amplitude and frequency, probably due to the action on M2 subtype receptors. Since the effects of this compound were stable in time with prolonged application and reversible with muscle washout, there are robust reasons to assert that compound **1** is a promising substance to be used in future drug development.

CONCLUSIONS

In this study, the effect of compound **1** (0.1–50 μM), whose ability to activate mAChRs and induce acetylcholine-induced and spontaneous contractions of rat *caecum* was predicted by *in silico* methods, was investigated, and the influence of this compound on the mechanokinetic parameters of the contraction-relaxation processes was studied.

It was found that compound **1** (1 μM) after short-term (5 min) application to the SM causes a significant increase in acetylcholine-induced contractions; such activation is eliminated by preincubation of SM with AF-DX 116 (an inhibitor of the M2 receptor subtype). This substance enhances spontaneous *caecum* motility in a time-dependent and dose-dependent manner; significant effects of increasing force, speed and impulse parameters are observed under the influence of its higher concentrations (1–50 μM). All studied concentrations of compound **1** significantly increase the frequency of spontaneous contractions in a dose-dependent manner, indicating the activation of the ICC pacemaker activity. The effects of compound **1** are stable for at least one hour of application to the *caecum* and are reversible and significantly eliminated by SM washing. There is a strong possibility that compound **1** activates M2 subtype mAChRs and can be used for drug development in future.

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COMPLIANCE WITH ETHICAL STANDARDS

C**onflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Human Rights: This article does not contain any studies with human subjects performed by any of the authors.

Animal Studies: All international, national and institutional guidelines for the care and use of laboratory animals were followed.

AUTHOR CONTRIBUTIONS

Conceptualization, [T.O.; S.S.; B.V.; N.A.]; methodology, [O.T.; V.I.; S.S.; B.V.; P.A.; F.T.; S.O.; B.A.; N.O.]; investigation, [O.T.; V.I.; S.S.; B.V.; P.A.; F.T.; S.O.; B.A.; N.O.]; data analysis, [O.T.; V.I.; F.T.; B.A.]; writing – original draft preparation, [O.T.; V.I.; S.S.; B.V.; P.A.; F.T.; S.O.; B.A.; N.O.]; writing – review and editing, [O.T.; V.I.; S.S.; B.V.; P.A.; F.T.; S.O.; B.A.; N.O.]; visualization, [O.T.; B.V.]; supervision, [O.T.; B.V.; S.S.; N.O.]; project administration, [O.T.; B.V.; S.S.; N.O.]; funding acquisition, [-].

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8-[(4-БЕНЗИЛПІПЕРАЗИН-1-ІЛ)МЕТИЛ]-3-(2-ХЛОРОФЕНІЛ)-7-ГІДРОКСИ-ХРОМЕН-4-ОН – АКТИВАТОР СКОРОЧУВАЛЬНОЇ АКТИВНОСТІ ГЛАДЕНЬКИХ М'ЯЗІВ ТОВСТОГО КИШЕЧНИКА З ВЛАСТИВОСТЯМИ ОБОРОТНОГО М2-ХОЛІНОМІМЕТИКА

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Обґрунтування. Низка патологій (таких як цукровий діабет, хвороби Паркінсона і Альцгеймера, розсіяний склероз тощо) супроводжуються дегенерацією холінергічних нейронів, які є ключовими регуляторами скорочувальної функції стінок ШКТ, призводячи до атоній і парезів. Ефективною стратегією нормалізації недостатності скорочувальної функції вісцеральних ГМ є використання лікарських засобів – селективних агоністів мускаринових ацетилхолінових рецепторів (mAChRs) М2 підтипу. Висока подібність структури агоніст-зв'язувальних сайтів різних підтипів mAChRs є причиною проблем зі створенням селективних лігандів цих рецепторів. Натепер надзвичайно нагальною є необхідність розробки селективних агоністів рецепторів М2 підтипу як фармакологічних інструментів для лабораторних досліджень і перспективних лікарських засобів.

Метою роботи було дослідження дії сполуки 8-[(4-бензилпіперазин-1-іл) метил]-3-(2-хлорофеніл)-7-гідрокси-хромен-4-он (сполука **1**), для якої методами *in silico* було передбачено здатність до активації mAChRs, на скорочувальну активність кільцевих гладеньких м'язів *caecum* щурів.

Матеріали та методи. Дослідження проводили на щурах. Скоротливу активність досліджували тензометрично в ізометричному режимі на препаратах кільцевих гладеньких м'язів сліпої кишки (*caecum*) щурів лінії Вістар. Кінетичні властивості окремих спонтанних скорочень ГМ препаратів здійснювали відповідно до методу багатопараметричного механокінетичного аналізу з розрахунком механокінетичних параметрів фаз скорочення і розслаблення: часових (τ_0, τ_C і τ_R), силових (F_{max}, F_C та F_R), швидкісних (V_C і V_R) та імпульсних (I_{max}, I_C та I_R). Аналіз кінетичних властивостей скорочувальних ефектів ацетилхоліну проводили з розрахунком нормованих максимальних швидкостей фаз скорочення (V_{nc}) та розслаблення (V_{nr}).

Результати. У роботі встановлено, що сполука **1** спричиняє підвищення амплітуди ацетилхолін-індукованих скорочень. Цей ефект усувається інкубацією ГМ з інгібітором mAChRs М2 підтипу AF-DX 116.

Виявлено, що сполука **1** (0,1–50 мкМ) також має здатність дозозалежно суттєво активувати функціональну активність ГМ товстого кишечника, збільшуючи силу і частоту спонтанних скорочень, а також їхні механокінетичні параметри.

Встановлено, що тривала дія (упродовж 1 години) сполуки **1** (0,1 мкМ) на гладенькі м'язи призводить до суттєвого збільшення амплітуди і частоти спонтанних

скорочень, а ці ефекти мають тенденції до виходу на стаціонарний режим після 40 хв її дії. Також ефект сполуки був оборотним.

Висновки. Сполука **1** активує скорочувальну активність кільцевих гладеньких м'язів товстого кишечника та проявляє властивості М2-холіноміметика.

Ключові слова: гладенькі м'язи товстого кишечника, мускаринові ацетилхолінові рецептори М2 підтипу, ацетилхолін, спонтанні скорочення, механокінетичний аналіз

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