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Auramine-O Potentiates the Duodenal Smooth Muscle Contraction Through Promotion of Cholinergic Signalling Pathway

Ayesa Khatun[1], Sandhi Paul[2], Sourapriya Mukherjee[1,3], Goutam Paul[1]

[1]Molecular Neurotoxicology Laboratory, Department of Physiology, University of Kalyani-741235, West Bengal, India.
[2]Ashiyan Medical College, University of Dhaka, Dhaka-1219, Bangladesh.
[3]Department of Physiology, KPC Medical College, Kolkata-700032.

Corresponding Author: Goutam Paul

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ABSTRACT

Auramine-O (AO), a synthetic dye belonging to the diarylmethane group, is extensively used in various industrial applications and is used as food additive. Exposure to AO in humans typically occurs through the ingestion of food products tainted with this synthetic colorant. Therefore, this study aimed to examine the effects of AO on the motor functions of the duodenum—a critical segment of the small intestine responsible for small intestinal motility. To evaluate the effect of AO on duodenal visceral smooth muscle (dVSM) motor activity, *ex vivo* recordings of duodenal movements were conducted using an isotonic transducer (IT-2245) connected to RMS Polyrite D. Rats exposed to AO showed a significant increase in the amplitude of duodenal contractions as compared to control rats in a dose dependent manner. From the tracings, it can be hypothesised that the potentiation of the contraction of the dVSM by AO might be due to facilitation of the activity of excitatory cholinergic myenteric efferents and/or inhibition of the activity of inhibitory nitrergic and/or adrenergic intrinsic myenteric efferents that innervates the SiVSM. Moreover, to investigate the probable neurocrine mechanisms in AO-induced enhancement of the duodenal visceral smooth muscle (dVSM) contractions, the movement of duodenum was recorded in response to application in combination with AO and cholinergic agonists (Acetylcholine) and antagonists (Atropine) respectively. AO exhibited a synergistic enhancement of duodenal visceral smooth muscle (dVSM) contractions in the presence of acetylcholine (ACh), whereas the AO-induced augmentation of dVSM contraction was significantly counteracted following pre-treatment with atropine, the muscarinic antagonist. In conclusion, it is suggested that AO enhances the motor functions of the SiVSM by augmenting the contractions of the SiVSM through cholinergic signalling pathway. These observations provide new insight into auramine's pharmacodynamic behaviour and raise critical concerns about its potential to disrupt normal gastrointestinal motility when ingested through contaminated food.

Keywords: Auramine-O, motor functions, small intestinal visceral smooth muscle, acetylcholine, atropine, cholinergic signalling pathway.

INTRODUCTION

Auramine-O (AO) ($C_{17}H_{22}ClN_3$), is a synthetic diarylmethane dye commonly used as a fluorescent stain in microbiological laboratories and as a component in textile, paper, leather, and cosmetic industries. It is a cationic dye and is often used in combination with other dyes like Rhodamine B for the staining of acid-fast bacteria in Ziehl–Neelsen staining procedures (McCarter & Robinson, 1994). Although industrially significant, AO is classified as a potential carcinogen and has raised considerable health concerns due to its unregulated use in food and cosmetic products, particularly in developing countries (EFSA, 2015). Auramine-O has been detected in food items, including turmeric powder, sweets, and pickled products, especially in informal sectors or local markets. Such unauthorized usage exposes humans through oral ingestion, which remains the primary route of exposure. Additional routes include dermal absorption through handling of contaminated textiles or cosmetics and inhalation in industrial settings (Chung, 2016). This unintentional ingestion of AO-contaminated food products poses a significant public health risk, particularly due to its lipophilic nature, which facilitates systemic absorption and accumulation.

The toxicokinetic of Auramine-O suggest that once absorbed, it undergoes hepatic metabolism, leading to the generation of reactive intermediates capable of inducing oxidative stress, DNA damage, and cytotoxicity (Parodi et al., 1982). Animal studies have shown that AO exhibits a wide tissue distribution, with notable accumulation in the liver, kidneys, lungs, and gastrointestinal tract. It is excreted through bile and urine. In toxicological studies on animals, AO has been linked to a range of adverse effects. Chronic exposure in rats and mice has shown hepatotoxicity, nephrotoxicity, neurotoxicity, and carcinogenic potential, particularly hepatocellular carcinomas (IARC, 1987). Sub-lethal doses have also been associated with alterations in intestinal morphology and gastrointestinal motility, although comprehensive studies on the gut smooth muscle are still limited. In human studies, although direct experimental evidence is sparse due to ethical constraints, epidemiological data and case reports from regions where AO is used illegally in food processing suggest correlations with gastrointestinal disturbances, allergic reactions, and possible hepatobiliary complications. Long-term exposure has raised suspicions of its role in gastric and liver cancers, although conclusive causation is yet to be established due to confounding exposures (Chung, 2016).

The gastrointestinal (GI) tract is a highly coordinated system that relies on smooth muscle motor functions to facilitate the movement that helps in digestion, and absorption of nutrients. The rhythmic contractions of the intestinal smooth muscles are regulated by an intricate interplay of enteric neurons, neurotransmitters, and intrinsic myogenic mechanisms. While the toxic and carcinogenic properties of Auramine-O have been widely explored, its pharmacological or physiological effects on the gastrointestinal tract, particularly on small intestinal smooth muscle contractions, remain under-investigated. Therefore, this study aimed to investigate the effect of Auramine-O on the motor functions of the small intestinal visceral smooth muscle (SiVSM) in rats and to understand the probable neurocine mechanism involved in it.

MATERIALS & METHODS

Chemicals and Reagents

All reagents and chemicals used in this study were of analytical grade. The test compound, Auramine O (≥98% purity), was obtained from Sigma-Aldrich. Other chemicals, including Acetylcholine chloride (ACh), Atropine sulfate (a muscarinic receptor antagonist), sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride ($MgCl_2$), calcium

chloride ($CaCl_2$), sodium bicarbonate ($NaHCO_3$), sodium dihydrogen phosphate (NaH_2PO_4), and glucose, were procured from E. Merck, India. All solutions were freshly prepared and gassed with 95% O_2 and 5% CO_2 before use.

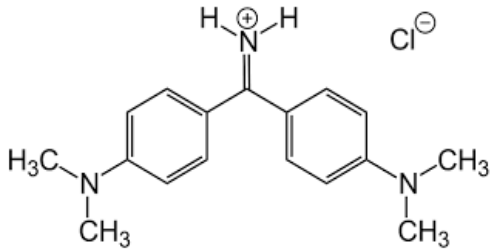

Figure 1: Chemical Structure of Auramine-O

Experimental Animals

Adult female albino rats of the Sprague Dawley strain, weighing approximately 130–150 grams and aged 2–3 months, were used as the experimental model in this study. The animals were housed in the departmental animal care facility at a controlled room temperature of 25–27°C, maintained under a 12-hour light-dark cycle. They were provided with standard laboratory chow and water ad libitum. All experimental procedures involving animals were conducted in strict accordance with the ethical guidelines approved by the Animal Ethics Committee of the University of Kalyani.

Experimental Design

The animals were treated to different exposure conditions as mentioned in Table 1.

Table 1: Experimental Setup for the study

Groups	Exposure condition
Set 1	Application of graded doses of AO (4, 8, 12, 16 µM) on the duodenal segments
Set 2	Application of single dose of ACh (0.01 µM) on the duodenal segments
Set 3	Application of 8 µM AO on duodenal segments pretreated with ACh
Set 4	Application of single dose of Atropine (1 µM) on the duodenal segments
Set 5	Application of 0.01 µM ACh on duodenal segments pretreated with Atropine (1 µM)
Set 6	Application of 16 µM AO on duodenal segments pretreated with Atropine (1 µM)
Set 7	Application of 16 µM AO on duodenal segments pretreated with Atropine (0.02 µM)

Animal Sacrifice

The animals selected for the experiment were subjected to overnight fasting prior to sacrifice to standardize physiological conditions. Cervical dislocation was employed as the method of euthanasia to ensure minimal pain and distress, in accordance with the ethical guidelines approved by the Animal Ethics Committee of the University of Kalyani.

Recording of the Movement of the Duodenum

To evaluate the spontaneous motility of the duodenal visceral smooth muscle (dVSM) ex vivo, an approximately 3 cm long segment of duodenum was carefully isolated and suspended vertically in an organ bath containing 40 mL of freshly prepared Tyrode's solution. The tissue segment was mounted using two stainless steel hooks—one at each end—pierced through the duodenal segment. The Tyrode's solution used had the following composition (per litter): NaCl – 8.0 g, KCl – 0.2 g, $CaCl_2$ – 0.2 g, $MgCl_2$ – 0.1 g, NaH_2PO_4 – 0.05 g, $NaHCO_3$ – 1.0 g, and dextrose – 1.0 g, adjusted to a pH of 7.4. To maintain tissue viability, a continuous oxygen supply was delivered near the tissue via an oxygen bubbler at a rate of 2–3 air bubbles per second. The organ bath temperature was precisely maintained at 37 ± 0.5°C using an automatic thermostat integrated with a Dale's apparatus. For recording contractile activity, the lower hook was fixed to the base of the organ bath, while the upper hook was connected to the lever arm of an isotonic transducer (Model IT-2245). The transducer was interfaced with a data acquisition system and analysis software (RMS Polyrite-D, RMS, Chandigarh, India) to continuously monitor and record the mechanical activity of the duodenal

segment. Each tissue segment was allowed to equilibrate for at least 35 minutes under the experimental conditions and was washed repeatedly with fresh Tyrode's solution to eliminate residual metabolites. Following stabilization, the isotonic contractions arising from the spontaneous rhythmic activity of the tissue were recorded continuously, and the responses to graded concentrations of Auramine-O (AO) and specific pharmacological blockers were subsequently analyzed.

STATISTICAL ANALYSIS

Data from each experimental group were expressed as mean ± standard error of the mean (SEM). The frequency and amplitude of the recorded duodenal movements were analyzed to calculate the force of contractions. For functional assessments, the values obtained from treated preparations were presented as percentage changes relative to their respective basal (control) values. One-way analysis of variance (ANOVA) was employed to determine statistical differences between experimental groups using GraphPad Prism version 8. A p-value less than 0.05 ($P < 0.05$) was considered statistically significant.

RESULTS AND DISCUSSION

Effect of graded doses Auramine-O (AO) on spontaneous duodenal contractions *ex vivo* in rats

To evaluate the impact of Auramine-O (AO) on the contractile activity of duodenal visceral smooth muscle (dVSM), *ex vivo* recordings of duodenal motility were performed using graded concentrations of AO in single-dose acute exposure experiments. Analysis of the contractile tracings revealed that, Auramine-O (AO) at increasing concentrations significantly enhanced both the frequency and amplitude of spontaneous contractions in a dose-dependent manner compared to control groups of rats (Figure 2 and Figure 3).

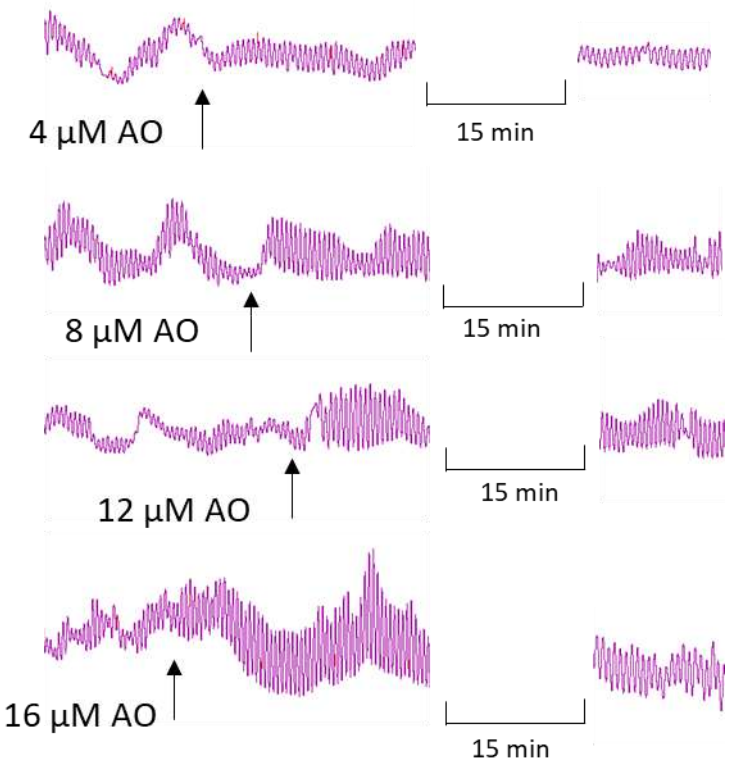

Figure 2. Tracings showing representative records of the effect of graded concentrations of Auramine-O on the isolated duodenal segment in order to examine the effect of Auramine-O on the motor functions of the SiVSM in rat *ex vivo* obtained with an isotonic transducer coupled to RMS Polyrite-D.

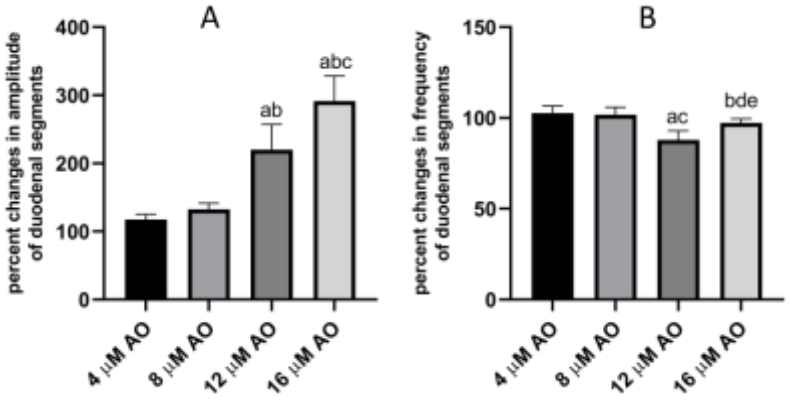

Figure 3. Bar diagrams showing the percent changes in the amplitude (A) and frequency (B) of contraction of the duodenum in AO exposed groups (4 µM, 8 µM, 12 µM, 16 µM) compared to control. The data were represented as mean ± SEM for all the groups. [a]p<0.0001vs. 4 µM AO, [b]p< 0.0001 vs. 8 µM AO and [c]p<0.0001 vs. 12 µM AO (A). [a,b]p<0.0001, 0.01 vs. 4 µM AO, [c,d]p< 0.0001, 0.05 vs. 8 µM AO and [e]p<0.0001 vs. 12 µM AO (B).

Effect of Auramine O on the Movement of Isolated Duodenum Pre-Incubated with Acetylcholine

The influence of cholinergic intrinsic myenteric efferents was elucidated in order to examine the probable Neurocrine mechanisms underlying in the AO induced potentiation of the contractions of the dVSM. The movement of the duodenum *ex vivo* was recorded in response to the application of acetylcholine, a cholinergic agonist; and AO in presence of ACh, in order to examine the cholinergic myenteric influences in the AO induced potentiation of the contractions of the dVSM. The tracings (Figure 3) indicate that pretreatment of duodenal segments with ACh increased the degree of potentiation of the dVSM contraction in comparison to AO alone. The synergistic potentiation of AO and ACh together suggests that AO might facilitate the cholinergic myenteric efferents which in turn augments the contractile activity potentiates the contractions of the dVSM. The intrinsic cholinergic myenteric efferents are principally excitatory to the contractions of the visceral smooth muscle situated at the muscularis externa layer of the duodenal wall, as they produce the excitatory neurotransmitter acetylcholine. This result suggests that AO probably sensitize muscarinic receptors or enhance/upregulate intracellular cholinergic signalling (e.g., increased IP_3 or Ca^{2+} mobilization) (Figure 4 and Figure 5).

Effect of Auramine O on the Movement of Isolated Duodenum Pre-Incubated with Atropine

Further, to determine the influence of cholinergic myenteric efferents in the AO induced potentiation of the contraction of the dVSM, the movement of the duodenum, a representative and initial part of the small intestine, was recorded *ex vivo* in response to the application of AO in combination with atropine, a cholinergic antagonist (cholinergic receptor blocker). The tracings showed that atropine alone initially inhibits the dVSM contractions, although this inhibitory action does not last very long. In contrast, when AO applied in atropine pre-treated duodenal preparations, we have found significant counteraction of the AO induced potentiation of the dVSM contraction, as observed through comparison with the potentiation of the dVSM contraction exhibited by AO alone (Figure 4 and Figure 5).

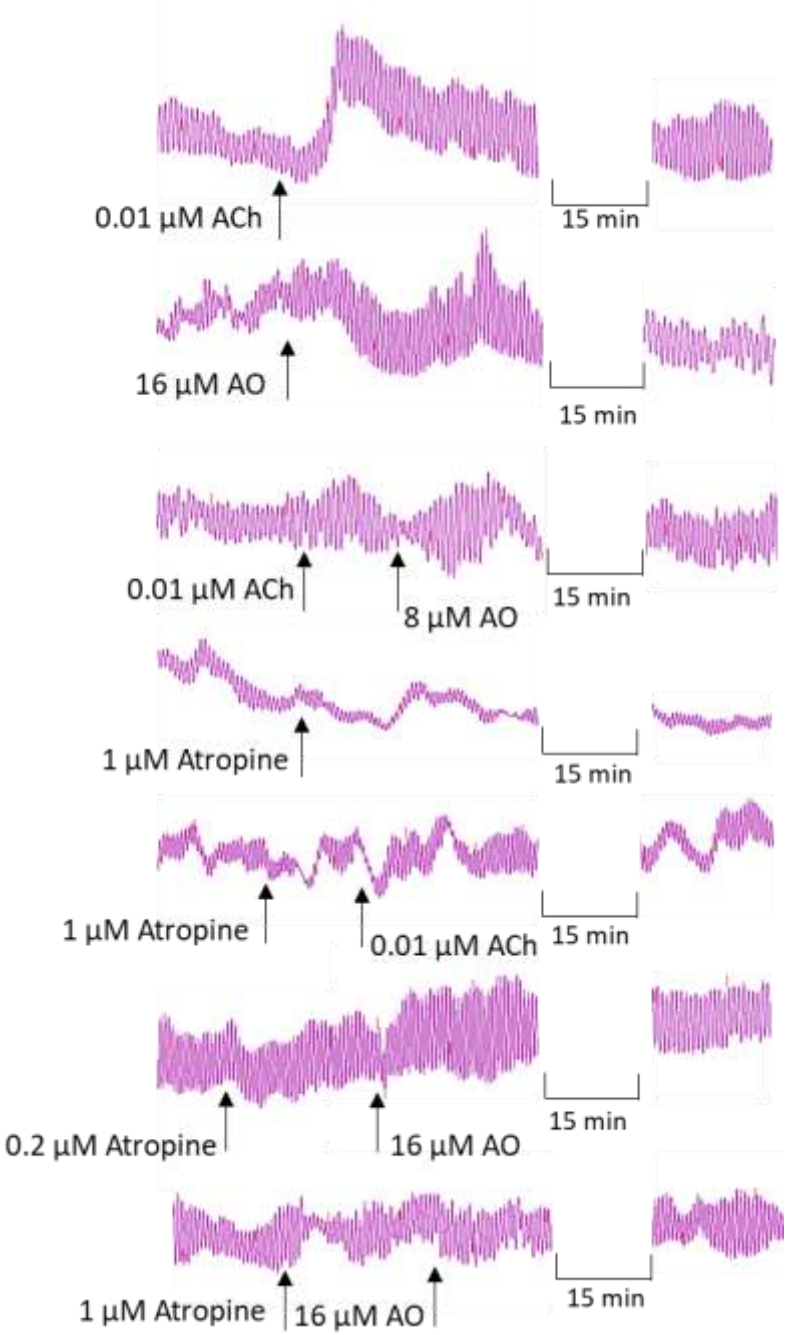

Figure 4. Representative tracings showing the effects of AO in combination with cholinergic agonist and cholinergic antagonist on the movement of duodenum *ex vivo* of rat in single dose acute exposure in order to examine the involvement of cholinergic signalling pathway in AO induced inhibition of the contraction of the duodenal visceral smooth muscle.

From these findings, it could be suggested that AO potentiates the contractions of the dVSM through facilitation of the activity of intrinsic cholinergic myenteric efferents that releases excitatory neurotransmitter, ACh at the synapse enpassant neurotransmission. This causes activation of G-protein coupled receptors that causes hydrolysis of Phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), that causes increase in the intracellular calcium level which in turn binds with the calmodulin and promotes contraction of the smooth muscles of the small intestine (Figure 6).

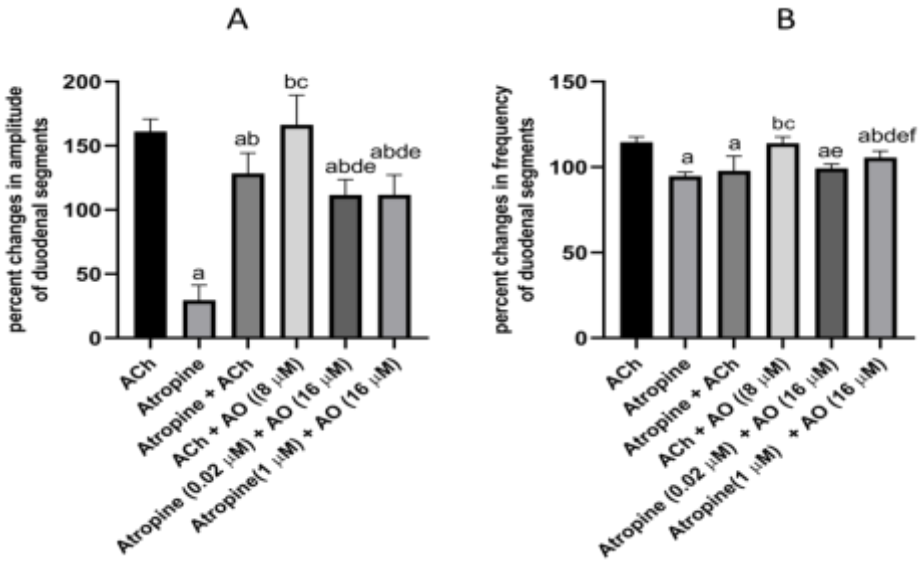

Figure 5. Bar diagrams showing the percent changes in the amplitude (A) and frequency (B) of contraction of the duodenum in response to the application of AO in presence of ACh and Atropine (Cholinergic agonist and antagonist). The data were represented as mean ± SEM for all the groups. [a]p<0.0001vs. ACh, [b]p< 0.0001 vs. Atropine and [c,d]p<0.0001, 0.05 vs. Atropine + ACh, [e]p<0.0001 vs. ACh +AO (8 µM) (A). [a]p<0.0001 vs. ACh, [b]p< 0.0001 vs. Atropine, [c,d]p<0.0001, 0.001 vs. Atropine + ACh, [e]p<0.0001 vs. ACh+AO (8 µM), [e]p<0.01 vs. Atropine (0.02 µM) +AO (16 µM) (B).

Auramine-O may act as a cholinomimetic compound—either directly binding to muscarinic receptors or increasing endogenous acetylcholine availability probably through inhibition of acetylcholinesterase or enhancement of neural release. The exact mechanism remains to be elucidated but could involve interaction with M_3 muscarinic receptors, which mediate contraction in GI smooth muscle.

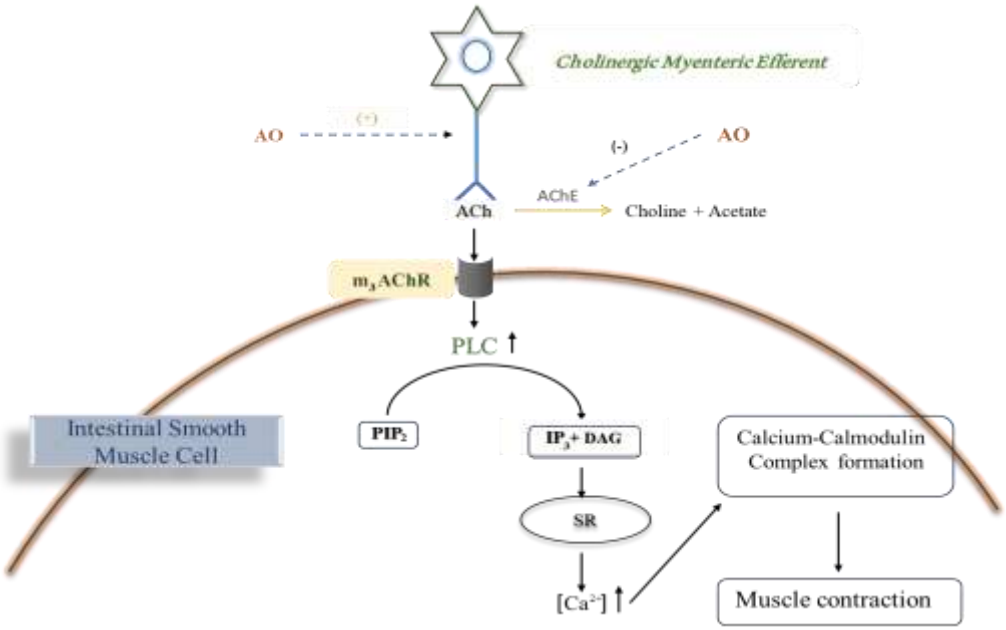

Figure 6. Schematic representation showing the probable mechanisms involved in the facilitation of the contraction of the dVSM by AO. AO- Auramine-O; ACh-acetylcholine; AChE- Acetylcholinesterase; [Ca^{2+}]-Intracellular calcium concentration; PIP_2 - Phosphatidylinositol 4,5-bisphosphate; PLC- Phospholipase C; IP_3-inositol 1,4,5-trisphosphate; DAG- Diacylglycerol. +↑indicates facilitation; -, indicates inhibition., indicates increase in production/activity.

CONCLUSION

This study reveals that Auramine-o significantly facilitates the duodenal smooth muscle contraction by increasing amplitude and frequency of contractions likely via stimulation of cholinergic muscarinic pathways. The findings provide new insights into the pharmacodynamic actions of auramine-o and raise concerns about its physiological impact when ingested through food adulterated with Auramine-O.

Declaration by Authors

Ethical Approval: Approved

Conflict of Interest: No conflicts of interest declared.

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