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Research Article

ANTAGONISTIC EFFECT OF RUTHENIUM RED ON ACETYLCHOLINE INDUCED CONTRACTIONS

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ABSTRACT

Ruthenium red (RR) is an inorganic dye used as a coloring agent, an indicator and for detecting pharmaceutical compounds. It is also used as a biological stain used to stain poly-anionic molecules such as pectin and nucleic acids for light microscopy and electron microscopy. Aim: The present study aimed to determine the potential of RR antagonistic effect against Acetylcholine (ACh) induced contractions using isolated frog's rectus abdominis muscle. This gives us information about the potential use of RR as a skeletal muscle relaxant property and can be used in the treatment of spastic disorders and to find potent muscle relaxant. Methods: Graded dose response of the ACh (contractions) on frog's rectus abdominis muscle was recorded to identify the sub-maximal dose. Then sub-maximal dose (8µg) along with gradual increase in RR dose (10, 20, 40, 80µg etc.,) was given to determine the effect of RR on contractions induced by ACh. PA_2 value of the RR against ACh was determined. Results**:** From the results it found that RR blocks the sub-maximal response at a dose of 640µg. The sub-maximal response of ACh was regained after RR treatment. PA_2 value of RR against ACh induced contractions on frog's rectus abdominis muscle was found to be 501.1µg. Conclusion: Finally, it was concluded that RR acts as a reversible blocker (antagonist) on nicotinic muscle receptors (N_MR_S) present on the skeletal muscle surface. Hence, it has potent skeletal muscle relaxant property.

Keywords: Antagonistic effect, Ruthenium red and acetylcholine

INTRODUCTION

Acetylcholine(ACh) acts as an excitatory neurotransmitter at neuromuscular junctions in skeletal muscle.[1] Skeletal muscle cells are excitable and are subject to depolarization by the neurotransmitter ACh, released at the neuromuscular junction by motor neurons.[2] The two main classes of ACh receptors are nicotinic receptors (nAChRs) which is stimulated by nicotine (agonist) and muscarinic receptors (mAChRs) which is stimulated by muscarine(agonist). Nicotinic AChRs are ionotropic receptors, permeable to sodium, potassium, and calcium ions. They are stimulated by nicotine and ACh which are not structurally similar compounds. The nAChRs are of two main types, muscle-type and neuronal-type. The former can be selectively blocked by curare and the later by hexamethonium. The main location of nAChRs is on motor end plates of muscle, on autonomic ganglia (both sympathetic and parasympathetic), and in the CNS.[3][4][5] Hyperactivity of the motor neurons or excess release of ACh leads to spastic disorders.[6]

Ruthenium red, also known as ammoniated ruthenium oxychloride**,** $[(NH_3)_5Ru\text{-}O\text{-}Ru(NH_3)_4\text{-}O\text{-}Ru(NH_3)_5]^{6+}$, is a biological stain used to stain polyanionic molecules such as pectin and nucleic acids for light microscopy and electron microscopy.[7] RR has also been used as a pharmacological tool to study specific cellular mechanisms. Selectivity is a significant issue in such studies as RR is known to interact with a large number of proteins.[8] It is also used in radiotherapy of eye tumors, mainly malignant melanomas of the uvea.[9] Ruthenium-centered complexes are being researched for possible anticancer properties.[10] RR protects HepG2 cells overexpressing CYP2E1 against acetaminophen cytotoxicity.[11] It is also reported that RR inhibits intracellular calcium release in the neuromuscular tissue of mammals.[12][13] Benzalkonium chloride, a preservative in eye drops was also found to be antagonistic to nicotine induced contractions.[14] Hence the present study aimed to determine the effect of RR on ACh induced contractions using frog's rectus abdominis muscle preparation. This gives us information about the potential use of RR as skeletal muscle relaxant used in treatment of spastic disorders.

MATERIALS

Chemicals

RR procured from Sidhi Chemicals, Visakhapatanam, India. ACh procured from Sigma Aldrich, New York, USA. Sodium dihydrogen phosphate (NaH_2PO_4) was procured from Sd Fine-chem Ltd, Mumbai, India. Sodium chloride (Nacl), Potassium Chloride (KCl), Calcium Chloride ($CaCl_2$), Sodium bicarbonate ($NaHCo_3$) and Glucose were procured from Qualigens Fine Chemicals, Mumbai, India. And all other chemicals used in the study were of analytical grade.

Animals Frog (Rana tigrina)

Physiological Solution Frog's Ringer solution

Equipments & Instruments Sherrington recording drum, Students organ bath, Simple Lever, Lever holder and Aerator

METHODS

Preparation of Frog's Ringer solution

NaCl (6.5g), KCl (0.14g), $CaCl_2$ (0.12g) and glucose (2g) were accurately weighed and dissolved in distilled water and was made to 900 ml. $NaHCO_3$ (0.2g) dissolved in distilled water was added slowly to the above solution by mixing and made to 1 L by distilled water. The final pH of the solution was adjusted and maintained at 7.4 by the addition of sodium hydroxide.

Preparation of drug solutions:

Ruthenium Red solution: 5mg of RR powder was weighed and dissolved in 50 ml of saline solution (0.9% NaCl)This gives a concentration of 1000µg solution.

ACh solution: 10mg of ACh was weighed and dissolved in 100ml of 5% sodium dihydrogen orthophosphate solution.

Isolation & mounting of rectus abdominis muscle of frog

A frog (Rana tigrina) was pithed by inserting pithing needle into the occipito atlantic junction or foramen magnum and destroying the brain and spinal cord. The skin on the abdomen was removed and the rectus abdominis muscle was exposed. It was separated from other muscle attachments. A midline incision on the abdominal muscle was made from pelvic girdle to pectoral girdle and two muscle preparations were identified. Two threads were sewn to the top and bottom of each muscle preparation before detaching it from the body of the frog. The rectus abdominis muscle from the body of frog was detached and mounted in up-right position in the organ bath containing frog Ringer solution with a tension of 1g.The organ bath was aerated and the tissue was stabilized for 45 min, during which period the tissue was washed with fresh quantum of Ringer for at least four times.[15][16]

Study design

The graded dose responses due to ACh were recorded on the frog's muscle following 5 min time cycle with 30sec baseline, 90sec response time and subsequent three washings at an interval of each minute was followed. The muscle was left for relaxation until the lever comes to the baseline position during which the muscle was washed with fresh quantum of Ringer each time. Responses to increasing doses of ACh were recorded until ceiling response was observed. A response on the sensitive region of the DRC was selected such that it is about 75% of the ceiling response and it is taken as sub-maximal response. The muscle was treated with RR for 30 sec and then the sub-maximal response of ACh was recorded. The effect of initial dose of RR was seen on sub-maximal response of ACh. Similarly the doses of RR were increased gradually namely 10µg, 20µg, 40µg, etc. to find their influence on the response of sub-maximal dose of ACh. The sub-maximal response of ACh was repeated in the end to check the consistency in its response when tried alone. The graph was fixed with a fixing solution. The heights of the responses were measured and the results are given in the Table no 1 and 2. The Institutional Animal Ethics Committee approved the study protocol.

Determination of PA_2 value

PA_2 value is defined as the negative logarithm of the molar concentration of antagonist, in the presence of which double the dose of agonist is required to produce the same effect as produced in absence of antagonist.

PA2 = -log [B] +log {[A2]/ [A1]-1}

= - antilog M

[B] – molar concentration of antagonist

$[A_2]$ – EC_{50} of agonist in the presence of antagonist

$[A_1]$ - EC_{50} of agonist in the absence of antagonist

M - Molar concentration of antagonist

PA_2 value is useful in differentiating the nature of receptors involved in particular drug action. PA_2 value for antagonist is always same, irrespective of the agonist used. Higher the PA_2 value, more potent is the antagonist. An antagonist acting on the same receptor will have same PA_2 value in all the tissue or organ preparations.

Procedure to determine PA_2 Value

The dissection, mounting of rectus abdominis muscle and time cycle followed was made as described previously. Responses to increasing doses of ACh were recorded until ceiling response was observed. Two doses bearing 1:2 dose ratio and eliciting sub-maximal responses (A, 2A) were selected for PA_2 determination say 4 µg and 8 µg respectively. The tissue was standardized with the selected doses of ACh. A tissue was said to be standardized when it responds identically to the same dose of an agonist when repeated. The concentration due to the double dose of ACh (8 µg) was recorded in the presence of varying concentrations (10µg, 20 µg, 40 µg etc.) of RR. The response due to double the dose of ACh (8 µg) i.e., before adding, was considered as 100% response. The corresponding percent response to the double dose of ACh (8 µg) in the presence of varying concentrations of RR were determined. A graph was plotted representing negative logarithm of molar concentration (-log M) of RR employed along X-axis and % response along Y-axis. The –log M at 50% was read out from the graph directly (Fig.1) and is used to determine PA_2 value using the above formulae or RR. It corresponds to the % response obtained with half the dose of ACh.[17]

Statistical Analysis

Data was analyzed by using one way ANOVA followed by Dunnett's t-test for multiple comparisons. Values with $P<0.05$ were considered significant.[18][19]

RESULTS

Antagonistic effect

ACh produced dose dependent response on isolated rectus abdominis muscle of frog. The amplitude of the initial contractile response against a dose of 1 µg was 8mm. The ceiling response of ACh was found to be 92 mm at 16 µg. The sub-maximal dose of ACh selected was 8 µg. RR was found to block the sub-maximal response of ACh on frog skeletal muscle. The dose required to produce such effect was 640 µg. Results shown on Table 1 and percentage change of ACh response in the presence of RR on normal rectus abdominis muscle of frog (N=5) was showed in Table 2.

PA_2 Value determination

ACh produced dose dependent response on isolated rectus abdominis muscle of frog. The amplitude of the initial contractile response against a dose of 1 µg was 4mm. The ceiling response of ACh was found to be 86 mm at 16 µg. The two doses selected were 4 µg and 8 µg and their responses obtained were 19 mm and 43 mm respectively. RR was found to decrease the response of double dose of ACh (8 µg) to that of single dose (4 µg) on frog skeletal muscle. The dose required to produce such effect was 128 µg. From the graph, the -log M value at 50% response was found to be 2.7 (Figure 1) and the PA_2 value was calculated as 501.1 µg.

Table 1: Antagonistic effect of RR on ACh induced contractions (N=5)

Dose of RR (µg) + ACh 8 µg	Height of the response in different trials (mm)						Mean ± SD
	T_1	T_2	T_3	T_4	T_5	Mean	
Control (ACh 8 µg)	52	50	49	54	55	52.00	52.00 ± 2.91
10	36	33	32	40	42	36.60	36.60 ± 4.33
20	35	32	30	38	39	34.80	34.80 ± 3.83
40	34	30	29	37	38	33.60	33.60 ± 4.03
80	30	28	26	33	31	29.60	29.60 ± 2.70
160	26	24	20	30	27	25.40	25.40 ± 3.71
320	17	15	13	20	18	16.60	16.60 ± 2.70
640	8	6	4	10	9	7.40	7.40 ± 2.40

Note: N=5. Values are statistically significant at $P<0.05$ (ANOVA)

Table 2: Percentage change of ACh response in the presence of RR (N=5)

Dose of RR + ACh 8 µg (µg)	Percentage reduction of response at different trials (%)						Mean ± SD
	T_1	T_2	T_3	T_4	T_5	Mean	
10	-30.76	-34.00	-34.69	-25.92	-23.63	-29.61	-29.61 ± 4.89
20	-32.69	-36.00	-38.77	-29.62	-29.09	-33.07	-33.07 ± 4.15
40	-34.61	-40.00	-40.81	-31.48	-30.90	-35.38	-35.38 ± 4.65
80	-42.30	-44.00	-46.93	-38.88	-43.63	-43.07	-43.07 ± 2.92
160	-50.00	-52.00	-59.18	-44.44	-50.90	-51.15	-51.15 ± 5.30
320	-67.30	-70.00	-73.46	-62.96	-67.27	-68.07	-68.07 ± 3.87
640	-84.61	-88.00	-91.83	-81.48	-83.63	-85.76	-85.76 ± 4.06

Table 3: Logarithmic values of RR and corresponding percentage response (%R)

M (RR)	- log M	Response, R (mm)	% R
10 µg	-1	39	90.69%
20 µg	- 1.3010	38	88.37%
40 µg	- 1.6020	37	86.04%
80 µg	- 1.9030	37	86.04%
160 µg	-2.2041	29	67.44%
320 µg	-2.5051	24	55.81%
640 µg	-2.8061	6	13.95%

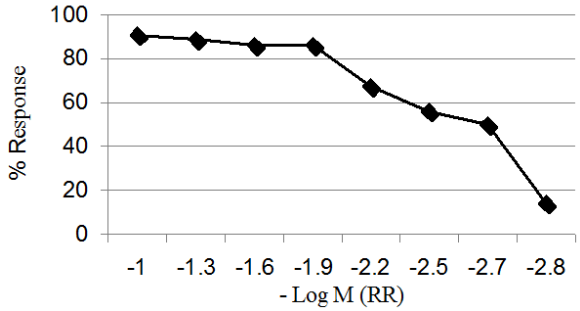

Fig. 1: Graph plotted –logM of RR Vs % Response

DISCUSSION

ACh acts on the nicotinic receptors at the motor end plate and produces depolarization which in turn leads to muscle contractions. RR blocked the contractions produced by ACh on frog's rectus abdominis muscle. The sub-maximal response was regained after the RR treatment. Thus from the results it is proved that RR acts as a reversible antagonist on nAChRs present on the surface of the skeletal muscle.

CONCLUSION

RR, an inorganic dye and staining agent blocked the action of ACh. Hence it has potential skeletal muscle relaxant property and can be used to treat spastic disorders.

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