Biochimica et Biophysica Acta, 772 (1984) 235–238
Elsevier

BBA Report

BBA 70156

CHOLINERGIC AGONISTS STIMULATE CALCIUM UPTAKE AND cGMP FORMATION IN HUMAN ERYTHROCYTES

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(Received October 17th, 1983)

Key words: Muscarinic receptor; Ca^{2+} uptake; cyclic GMP; Cholinergic agonist; (Erythrocyte membrane)

Human erythrocytes possess a muscarinic cholinergic receptor sensitive to cholinergic agonists which stimulate transient increases in calcium uptake and subsequent cyclic GMP formation. These phenomena can be blocked by atropine and EGTA. The cholinergic stimulation of cyclic GMP formation depends on Ca^{2+} uptake from external media. The effects of cholinergic agonists on the erythrocyte resemble their effect on calcium channels in nervous tissue. The cholinergic stimulation of Ca^{2+} uptake in erythrocytes may affect the calcium-sensitive mechanism involved in the shape, permeability and rigidity of these cells.

Vasoactive hormone and neurotransmitters have been reported to alter the deformability, osmotic fragility and gross membrane structure of the washed human erythrocyte [1]. Bovine erythrocytes show a change in structural arrangement when exposed to acetylcholine [2]. Human erythrocytes treated with carbamylcholine (carbachol), a cholinergic muscarinic agonist, develop and increase in the cation permeability [3], and a dramatic increase in uptake of ^{22}Na [4]. Addition of tetradotoxin to treated erythrocytes decreases the ^{22}Na uptake. This suggests that the erythrocyte membrane contains sodium channels resembling those involved in the action potential propagation in nerve muscle [4]. There is evidence that, in excitable cells, sodium influx triggers an increase in intracellular free calcium which, in turn, stimulates the formation of guanosine 3′,5′-cyclic-monophosphate (cGMP) [5]. Red blood cell intracellular calcium is rigidly maintained between 10 and 20 μmol/l of cells [6]. Incremental changes in calcium in erythrocytes affect their metabolic rates [7], contents of electrolytes [8], enzymatic activities [9], cell rigidity [10], cell shape [11] and possibly susceptibility to hemolysis [12].

Specific muscarinic cholinergic receptors have been demonstrated on the human erythrocyte membrane [13]. Quinuclidinyl benzilate, a muscarinic cholinergic antagonist, binds specifically to erythrocyte ghost and this binding is inhibited by atropine, another muscarinic antagonist. Furthermore, carbamylcholine, in presence of calcium, has been found to stimulate the formation of cGMP in human erythrocytes (unpublished observation). In an effort to explore the possible roles of this red blood cell muscarinic receptor in red blood cell physiology, we have examined the effects of cholinergic agonists on calcium flux and its effects on cGMP formation.

Studies for evaluating the effect of cholinergic stimulation on calcium flux was carried out in freshly drawn intact erythrocytes from healthy

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Abbreviation: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

volunteer males. The red blood cells were separated from the plasma, white cells and platelets by centrifugation at $800 \times g$ for 10 min at 4°C. The red blood cells were then washed with 10 ml of 10 mM Tris buffer (pH 7.4), with 140 mM NaCl, 5 mM KCl, 2 mM $MgSO_4$ and 5.6 mM glucose and suspended in the same buffer containing 1 mM $CaCl_2$ in a volume ratio of 1 : 4. 50 μl of 100 μM Ca^{2+} containing 0.2 μCi $^{45}Ca^{2+}$ were added to the suspension and incubated for 15 min at room temperature. This was found to be sufficient for labelled Ca^{2+} to equilibrate in the suspension. 0.5 ml aliquots of red blood cell suspension were removed prior to and at 10-s intervals after the addition of 100 μM carbachol (final concentration). Ca^{45} uptake was stopped by diluting the 0.5 ml aliquots with 5 ml ice-cold (4°C) Tris buffer (pH 7.4), containing 5 mM $CaCl_2$. This increased the external Ca^{2+} concentration and the slowed exchange due to cooling effectively trapped the Ca^{2+} in the cells. The cells were washed three times with 10 ml of this buffer to obtain cells free of radioactive Ca on the outside. The erythrocytes were then charred in a 210°C oven for 30 min, pulverized and extracted with 1 ml of 1 M HCl. The pH of the extract was brought to 7.0 with 1 M NaOH. Duplicate 100-μl aliquots of the extract were added to 10 ml aquasol and counted three times each in a beta liquid scintillation counter.

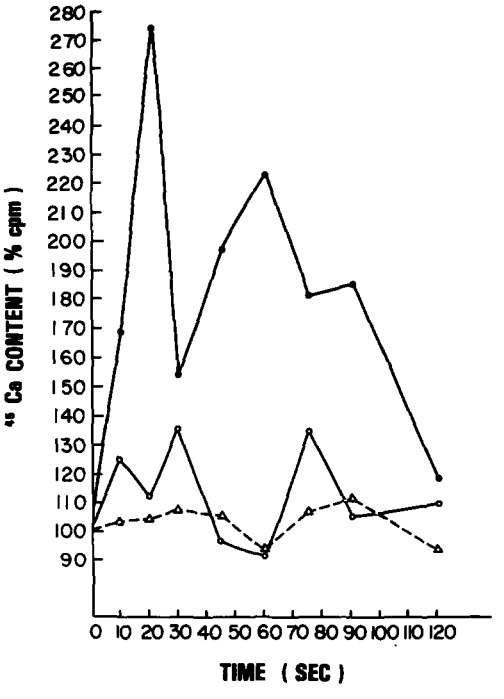

Fig. 1. ^{45}Ca uptake in human erythrocytes (RBC). O——O, Control (RBC + dilutant); ●——●, carbachol (100 μM) stimulated; △——△, carbachol (100 μM) stimulated + atropine (100 μM).

Carbachol, at 100 μM, stimulated ^{45}Ca uptake in erythrocytes obtained from normal males. Maximum uptake occurred between 20 and 35 s following addition of carbachol and gradually returned to baseline levels after approx. 2 min. As can be seen in Fig. 1, the uptake varies within 30% in the control non-treated erythrocytes, whereas, in the cells after exposure to carbachol the radiolabelled Ca increased over 250%. Similar increases in Ca uptakes were observed in nine separate experi-

TABLE I

CYCLIC GMP IN HUMAN ERYTHROCYTES

Human red blood cells prepared freshly were suspended in 10 mM Tris buffer (pH 7.4), with 140 mM NaCl, 5 mM KCl, 2 mM $MgSO_4$ and 5.6 mM glucose. $2 \cdot 10^5$ cells per ml were incubated at room temperature for 2 min with either saline or chemicals listed. The reaction was terminated by immersing the tubes in boiling water. To each sample 200 μl of 50% trichloroacetic acid was added and the mixture sonified for 30 s. The trichloroacetic acid was extracted with 10 ml water saturated ether (three times). The samples were then dried in a 210°C oven for 1 h. cGMP in each sample was determined by radioimmunoassay according to the method of Goldberg et al. [14].

	Conc. added (μM)	No. of samples	cGMP/$1 \cdot 10^6$ cells (pmol) Mean ± S.E.
Saline		7	0.41 ±0.035
* Carbachol	100	6	0.825 ±0.046
Atropine	100	4	0.40 ±0.020
Carbachol + atropine	100 100	6	0.44 ±0.017
EGTA	100	3	0.415 ±0.18
Carbachol + EGTA	100 100	4	0.425 ±0.015
Trifluperazine	100	4	0.405 ±0.015
Carbachol + trifluperazine	100 100	4	0.40 ±0.014
* Calcium	250	4	0.60 ±0.015
* Carbachol + calcium	100 250	3	1.285 ±0.05
* Ionophore A23187	50	3	0.8125 ±0.03

* $P < 0.01$ compared with the saline treated control, calculated using Student's t-test.

ments. Each experiment was evaluated individually since the time of maximum Ca^{45} content following carbachol stimulation did not coincide. The increase in ^{45}Ca uptake was abolished by addition of atropine ($1 \cdot 10^{-7}$ M), EGTA (50 μM), a Ca chelator, and trifluperazine (50 μM), a calmodulin inhibitor. These experiments suggest that muscarinic agonist binds to the acetylcholine receptor and thus enhancing the permeability of calcium into the erythrocyte.

To evaluate the role of calcium on the cyclic GMP production, cGMP content was determined by radioimmunoassay [14] in intact red blood cells. Fresh, whole human erythrocytes were incubated at room temperature with 100 μM carbachol, and in various combinations: 100 μM atropine, 100 μM EGTA, 100 μM trifluperazine, 250 μM $CaCl_2$ and 50 μM A23187 (a calcium ionophore), as shown in Table I. cGMP levels doubled after a 2 min exposure to carbachol. However, this increase was completely abolished when the incubation solutions contained atropine, EGTA or trifluperazine (Table I). cGMP content was unaffected by incubating with atropine, EGTA or trifluperazine alone. A calcium dependence of the carbachol-stimulated cGMP is suggested not only by the inhibitory effects of EGTA but the enhanced response on cGMP stimulation when additional Ca^{2+} was added to the incubation media (Table I) and marked stimulation with the Ca^{2+} ionophore, A23187. A comparison of the time-course of Ca^{2+} entry and cGMP formation is shown in Fig. 2. The early increase in Ca^{2+} uptake preceded an increase in cGMP formation. The fact that ca^{2+} uptake preceded cGMP formation in each experiment suggests a temporal relationship.

The results of this study strongly suggest that human erythrocytes possess a muscarinic cholinergic receptor sensitive to cholinergic agonists which stimulate transient increases in calcium uptake and subsequent cyclic GMP formation. The muscarinic agonists, carbachol, increases calcium uptake by approximately three times the control values. This uptake was blocked by the antagonist atropine, by chelation of Ca with EGTA, and by the calmodulin blocker trifluperazine. However, from these data it is difficult to determine if the action of trifluperazine is mediated through an inhibitory effect on calmodulin or

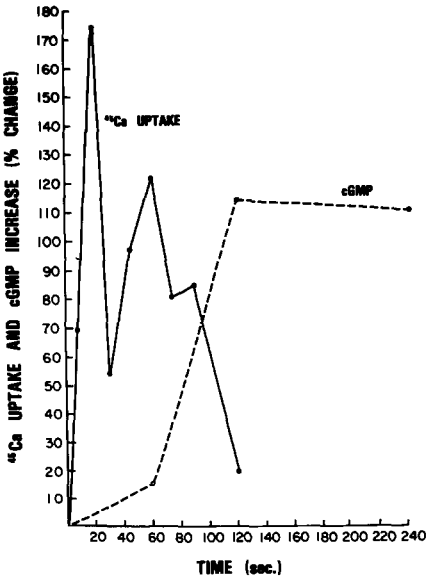

Fig. 2. ^{45}Ca uptake and cGMP formation in human erythrocytes after carbachol stimulation plotted against time.

if trifluperazine simply blocks calcium uptake. Although a slight stimulation of cGMP formation was observed in the absence of added calcium, a marked stimulation was obtained with the addition of 250 μM calcium to the incubating media. This suggests that the effects of calcium on cGMP formation exerted by muscarinic agonists are mediated by an influx of calcium from the media. This mechanism differs from that reported for the calcium-dependent adrenergic stimulation of band 2 phosphorylation in human erythrocyte, since the effects of adrenergic agents occur in the absence of extracellular calcium suggesting that this mechanism involves a mobilization of intracellular calcium stores by adrenergic agonists [15]. The cGMP formation reported in this study clearly followed the carbachol-stimulated calcium uptake and was dependent on calcium in the media as evidenced both by the inhibitory effect of EGTA and an enhanced effect observed with the addition of calcium to the media. The effects of carbachol on calcium uptake and cGMP formation in the red blood cell are reminiscent of its effects on calcium channels in neurons and neuroblastoma cells [16]. Dependence on external calcium for the cholinergic stimulation of cGMP formation appears to be a universal phenomena.

A characteristic of the muscarinic cholinergic receptor-mediated cGMP synthesis is the rapid onset and the short duration of the response [16]. This time-course is dependent upon extracellular Ca^{2+} or other agents that require Ca^{2+} to generate a cGMP response [16,17]. The muscarinic receptor of mouse neuroblastoma NIE-115 cells which mediates the cGMP response undergoes rapid desensitization [18] with a time course rapid enough to suggest that receptor inactivation is responsible for the decrease in cGMP synthesis [19]. Likewise, in our experiments with human erythrocytes, the rapid increase and decline of the calcium uptake were observed despite the continued presence of carbachol in the media. Whether this effect is due to desensitization of the cholinergic receptor or perhaps enhanced activity of the erythrocyte Ca^{2+}-ATPase in response to the calcium influx cannot be presently determined.

A large body of evidence has accumulated to show that changes in intracellular calcium concentration in erythrocytes have profound effects on numerous membrane and metabolic functions of these cells [7]. Calcium accumulation in erythrocytes has been shown to cause marked losses of ions with dehydration and changes in intracellular viscosity, resulting in limited cell deformability [20]. There is also evidence that calcium may exert a direct effect on membrane components to produce spherocytes independent of ATP depletion. Certainly, small incremental changes in intracellular calcium concentration may affect membrane protein phosphorylation [11], calmodulin mediated events [21], hemoglobin function and numerous glycolytic enzymes [22]. The exact mechanism by which carbachol stimulated calcium influx and cGMP formation and the potential functional changes in the erythrocyte awaits further investigation.

Acknowledgements

We thank Dr. Donald Butkus and Dr. Daniel G. Wright for their constant encouragement and advice.

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