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INHIBITORY EFFECT OF IONIZED FREE INTRACELLULAR CALCIUM ENHANCED BY RUTHENIUM RED AND *m*-CHLORO-CARBONYLCYANIDE PHENYL HYDRAZON ON THE EVOKED RELEASE OF ACETYLCHOLINE

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Abstract—In order to understand the relationship between the free ionized calcium concentration in the axon terminals and the transmitter release we have investigated the effect of ruthenium red (RuR) and *m*-chloro-carbonylcyanide phenyl hydrazon (CCCP), mitochondrial uncoupler agents on the liberation of acetylcholine from myenteric plexus of guinea-pig ileum. Both compounds are able to enhance intracellular free Ca^{2+}.

In the presence of RuR and CCCP the spontaneous release of radioactivity from isolated myenteric-plexus preparation, previously loaded with (methyl-3*H*)-choline chloride was significantly enhanced while that evoked by electrical stimulation was decreased. Atropine did not affect the electrically evoked release in the presence of CCCP, indicating that in the effect of CCCP the presynaptic muscarinic receptors mediated negative feedback modulation does not play any role. Our findings are consistent with the hypothesis that the enhanced level of intracellular free Ca^{2+} enhances the spontaneous while depresses the electrically evoked release of transmitter.

According to the calcium hypothesis originally proposed by Katz and Miledi, calcium plays a critical role in the process of excitation–secretion coupling in synaptic transmission [1–6]. It was proposed that the depolarisation of the nerve terminal opens Ca^{2+}-channels, calcium then enters the terminals down a steep electrochemical gradient. While the evoked release of transmitter is a Ca_0-dependent process, spontaneous release is relatively insensitive to extracellular Ca^{2+}. Nevertheless, the spontaneous release strongly depends on the intracellular level of free Ca^{2+}. Even in the absence of extracellular Ca^{2+}, increases in spontaneous release can be produced by agents which cause Ca^{2+} to leak from cellular storage sites such as smooth endoplasmic reticulum and mitochondrium [7–11] or by interfering with the active extrusion of intracellular calcium ions across the plasma membrane [12, 13] or by inhibiting Na^+/K^+-ATPase [14, 15]. The inorganic dye ruthenium red (RuR) which can penetrate into cells both *in vivo* and *in vitro* [16] is known to inhibit mitochondrial uptake of calcium thereby enhancing the free cytosolic Ca^{2+} concentration. The calcium ion concentration in the presynaptic terminal can be manipulated by CCCP as well, an inhibitor of calcium sequestration of mitochondria. Since the relationship between the free ionized calcium concentration in the presynaptic terminal and the transmitter release is not well defined, we have investigated the action of RuR and CCCP on the release of ACh from the longitudinal muscle strip together with Auerbach's plexus. This preparation had proved to be very useful for reliability measuring both resting and electrical stimulation evoked release of ACh [19]. Our results providing neurochemical evidence are consistent with the hypothesis that increased intracellular level of ionized calcium in the presynaptic terminal enhances the spontaneous while reduces the electrically evoked release of transmitter.

MATERIALS AND METHODS

Materials. (Methyl-3*H*)-choline chloride 15 Ci/mmol (Amersham International Ltd., U.K.), hemicholinium-3 (Sigma Chemical Co, St Louis, MO), quinine sulfate (Sigma Chemical Co, St Louis, MO) *m*-chloro-carbonylcyanide phenyl hydrazon (CCCP) (Calbiochem Behring Co.) and atropine methyl bromide (Sigma Chemical Co.) were purchased. Pure ruthenium red was obtained by crystallization [19]. Guinea-pigs of both sexes weighing 300–400 g were used. Preparation of myenteric plexus-longitudinal muscle strip was carried out as described by Paton and Vizi [20]. The tissues were incubated at 37° in Krebs solution (NaCl 113, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, $KHPO_4$ 1.2 and glucose 11.5 mmol/l) containing 4 μCi/ml methyl-3*H*-choline chloride (15 Ci/mmol). When Ca-excess (12.4 mM) was used the concentration of $NaHCO_3$ was reduced to 5 mmol/l in order to reduce precipitation. The bath was gassed with 95% O_2 + 5% CO_2 during both the incubation and the collection period. After the incubation period the tissues were perfused at a rate of 1 ml/min with Krebs solution containing 10 μM hemicholinium-3 to inhibit the reuptake of (3*H*)-choline originating from the hydrolysis of released (^{3}H)-ACh. In five cases the experiments were carried out in the presence of physostigmine (2 μM) and labeled choline and acetylcholine were

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separated by choline-kinase-technique as described previously [21]. Forty-five microliters of a solution of 300 mM glycin buffer (pH 0.8), 13.3 mM ATP and 80 mM $MgCl_2$ were added to 170 μl of the collected 5-ml samples in 5-ml Pyrex vials. The reaction was initiated by the addition of 100 μl of choline kinase (0.01 U/ml). Reaction mixture was incubated at 37° for 30 min. This amount of choline kinase is able to catalyze the phosphorylation of 50 nmol of choline to choline phosphate by ATP per minute (see Sigma Catalog, p. 227, 1983). At the end of the incubation period, the reaction is terminated by the addition of 300 μl of water and 4.5 ml of scintillation fluid, containing 3 mg/ml of sodium tetraphenylboron dissolved in 0.1 ml of isoamylethanol. Triton-X 100 was not added to the scintillation fluid. The vials were capped, shaken and counted. [^{3}H]Choline is completely converted to [phosphoryl-^{3}H]choline by choline-kinase and is extracted into the water phase. In contrast [^{3}H]ACh remains in the organic phase. Therefore, the radioactivity counted at this stage represents [^{3}H]ACh. After determining [^{3}H]ACh, 3 ml of Triton-X-100 were added to the vials and they were counted again. The radioactivity measured this way represents the total release ([^{3}H]choline + [^{3}H]ACh). Two types of collection method were used. In the case of RuR the tissues were not perfused, the bathing fluid (2 ml) was changed in every 10 min [19] and the radioactivity of the fluid was measured. When the effect of CCCP, atropine and Ca-excess on the evoked release of ACh was studied, perfusion technique [21] was used. Collection of fluid in 10-min fractions for RuR and 3-min fractions for CCCP began after preperfusion of 60 min. During the collection of the 3rd, 10th and 15th samples for RuR and the 3rd, 15th and 25th samples for CCCP tissues were stimulated (2 Hz, 1.0 msec, 360 shocks, supramaximal voltage). RuR or CCCP was added into Krebs solution between 60 and 120 min and 30 and 60 min, respectively. At the end of each experiment the radioactive content of samples as well as tissues was measured by liquid scintillation spectrometry. The effect of RuR and CCCP on electrically evoked release was expressed as the ratio of S_{N+1}/S_N in the presence and absence of the drug. The release of radioactivity was expressed as a percentage of the total (3*H*) present in the tissue at the onset of collection of the sample. Statistical analysis of data was carried out using Student's *t*-test. P values of less than 0.05 were considered significant. The means ± SEM of the obtained results are used throughout this paper.

RESULTS

The spontaneous release of radioactivity measured in 10-min samples from isolated longitudinal muscle strip, myenteric plexus preparation of guinea-pig ileum represented 1.9 ± 0.1% (N = 8) of the total radioactivity remaining in the tissue. Electrical field stimulation increased the release of radioactivity by 2637 ± 365 Bq/g (N = 8) above resting level. Three consecutive stimulations (S_1, S_2 and S_3) resulted in a relatively constant release of ACh: the ratios of S_2/S_1 and S_3/S_2 were 0.84 ± 0.09 and 0.79 ± 0.08, respectively (N = 4–4, S_3/S_1 = 0.66 ± 0.07). During resting 45.7 ± 4.6% of the radioactivity released was ^{3}H-ACh and at stimulation it was 91.6 ± 5.8% as evidenced by the separation technique. Figure 1 shows the effect of 100 μM RuR on spontaneous and electrically evoked release. While RuR significantly enhanced the amount of radioactivity spontaneously released, it significantly decreased the electrically evoked (S_2) release. S_2/S_1 ratio was reduced from 0.81 ± 0.07 (N = 8) to 0.20 ± 0.04 (N = 4; P < 0.01). After RuR wash-out, the spontaneous release was decreased to control level while 20 min after its removal the S_3/S_2 ratio was 0.94 ± 0.16 indicating an irreversible effect of RuR on evoked release (S_3/S_1 = 0.19 ± 0.02), compared to control value (0.66 ± 0.07); the difference is significant (P < 0.05, N = 4). CCCP is a potent mitochondrial uncoupler and has been shown to increase ionized calcium levels irreversibly. Perfusion with Krebs solution containing 0.5 μM CCCP (Fig. 2) produced a significant increase in the resting release (P < 0.01) of ACh and a progressive reduction in the evoked release of ACh as S_2/S_1 ratio was reduced from 0.81 ± 0.07 (N = 8) to 0.49 ± 0.03 (N = 6; P < 0.01). CCCP in a contraction of 5 μM reduced the S_2/S_1 ratio further from 0.81 ± 0.07 (N = 8) to 0.18 ± 0.03 (N = 4; P < 0.01) as illustrated in Fig. 2. The effect of CCCP on the evoked release of ACh was irreversible: after 20 min wash-out there was no recovery of ACh release (in Fig. 2). The increase of radioactivity release in response to CCCP (5 μM) administration or to electrical stimulation but in the presence of CCCP (5 μM) was mainly due to the enhancement of ^{3}H-ACh release (87.5 ± 9.6 and 91.3 ± 11.6%, respectively) as it was evidenced by the separation technique.

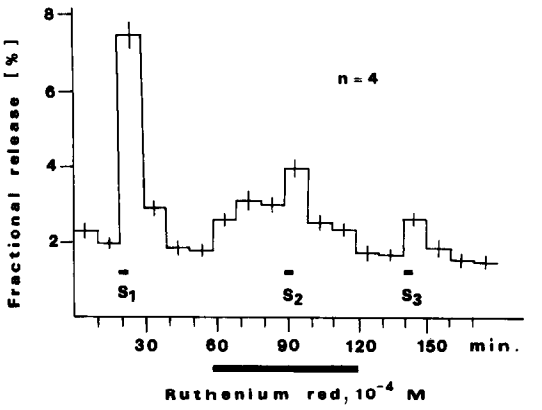

Fig. 1. Effect of ruthenium red in 10^{-4} M concentration on the release of radioactivity from myenteric-plexus of guinea-pig ileum previously labeled with [3*H*]-choline. Bathing fluid was changed in every 10 min. Horizontal bars represent the means, verticle ones represent SEM (N = 4).

It was investigated whether the decrease in the amount of electrically evoked release can partly be attributed to the muscarinic receptor mediated negative feedback mechanism elicited by the enhanced spontaneous release of ACh in response to CCCP administration. Atropine in 10^{-7} M concentration did not significantly influence the effect of CCCP on resting and electrically evoked release, although in itself enhanced the release evoked by stimulation

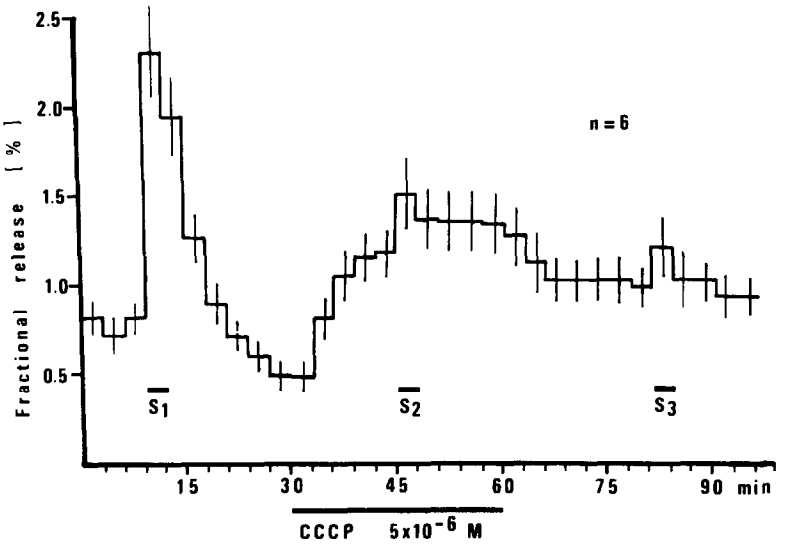

Fig. 2. Effect of *m*-chloro-carbonylcyanide phenyl hydrazon (CCCP) in a concentration of 5.10^{-6} M on the release of radioactivity from myenteric plexus of guinea-pig ileum previously labelled with [3*H*]-choline. The tissue was superfused at a rate of 1 ml/min. Samples were collected in 3-min fractions. Horizontal bars represent the means, vertical ones represent SEM (N = 6).

(Table 1). If the irreversible failure of electrically evoked release of ACh produced by CCCP were due to reduced calcium influx during the presynaptic action potentials, then raising the extracellular calcium concentration may overcome the inhibition of evoked release. In three experiments, the longitudinal muscle preparation was bathed with high-calcium (12.4 mM) Krebs solution. The elevated calcium did not prevent the effect of CCCP (Table 1). However, quinine sulphate, a blocker of Ca-activated K channels, partly prevented the effect of CCCP.

DISCUSSION

Preparation of myenteric plexus-longitudinal muscle strip labeled with ^{3}H-choline was used to study the effect of increase of intraterminal Ca concentration by RuR or CCCP on resting and evoked release of ACh. As shown by a choline kinase separative technique, the increase of the release of total radioactivity in response to electrical stimulation and to CCCP consisted mainly of ^{3}H-ACh.

The total concentration of Ca^{2+} in cells is similar to that of extracellular Ca^{2+} (10^{-3} M) but the concentration of free calcium ion in the cytosol is more than a thousandfold lower (10^{-7} M) because most of the Ca^{2+} in the cells is bound to other molecules or sequestered in mitochondria and other intracellular organelles. It means that there is an enormous gradient in the concentration of free calcium ion across the plasma membrane. Electrophysiological evidence has been obtained that RuR depresses the evoked release of transmitter while it increases the

Table 1. Effect of *m*-chloro-carbonyl cyanide phenyl hydrazon (CCCP) on the electrically evoked release

	Electrically evoked release of ^{3}H (S_2/S_1)	N	Significance
1. Control	0.81 ± 0.07	8	
2. CCCP, 0.5 μM	0.49 ± 0.03	4	2:1 $P < 0.01$
3. CCCP, 5.0 μM	0.18 ± 0.03	4	3:1 $P < 0.01$
4. CCCP, 5.0 μM + atropine, 0.1 μM	0.19 ± 0.03	3	4:3 $P > 0.05$
5. Atropine, 0.1 μM	2.21 ± 0.21	5	5:1 $P < 0.01$
6. CCCP, 5.0 μM + 12.4 mM Ca_0^{2+}	0.16 ± 0.05	3	6:3 $P > 0.05$
7. CCCP, 5.0 μM + quinine, 10 μM	0.52 ± 0.07	3	7:3 $P < 0.01$

The tissue was superfused at a rate of 1 ml/min. 3-min fractions were collected. Release of radioactivity by electrical stimulation (2 Hz, 1.0 msec, supramaximal voltage) was measured from myenteric-plexus of guinea-pig ileum previously labeled with (^{3}H)-choline. The results are expressed as means ± SEM. Atropine or quinine was perfused together with CCCP (see Fig. 2). Normal Krebs solution was changed for modified Krebs (Ca-excess) when CCCP was added into the fluid at 30th min. During resting 45.7 ± 4.6% of the radioactivity released is ^{3}H-ACh, at stimulation this value is 91.6 ± 5.8%.

spontaneous release from the neuromuscular junction [22] and from squid giant synapse [1, 13, 23]. It has been reported using neurochemical methods that RuR enhances veratridine-induced release of ^{3}H-noradrenaline [24] and inhibits the Ca^{2+}-dependent release of (3*H*)-GABA evoked by K^+-excess from the synaptosomal fraction of mouse brain [25, 26] presumably by raising the intracellular level of free Ca^{2+} [13]. This suggestion was substantiated by the finding that injection of EGTA into presynaptic terminals poisoned with cyanide produced transient increases in transmitter release [13]. It has also been reported that CCCP is able to release ACh from rat brain synaptosomes even in the absence of external calcium [10]. In the present work neurochemical evidence has been presented that ruthenium red and CCCP in concentrations of 100 μM and 0.5 μM, respectively, increase the spontaneous release, whereas decrease the electrically evoked release of ACh, though do not completely abolish it. Since these compounds significantly increase intracellular free Ca^{2+} [7, 27] a simple explanation for our observations is that a sustained rise in presynaptic intracellular calcium level exerts an inhibitory effect on electrically evoked release of ACh. Since in another calcium dependent release system it has been shown that in bovine adrenal medullary cells [28] the high intracellular level (5–100 μM) of ionized calcium is responsible for the inhibition of exocytosis, it seems feasible to suggest that the Ca_o-dependent, electrically evoked release of ACh is also depressed by intracellular ionized Ca [23]. Besides, it has been shown that RuR blocks the depolarization induced calcium entry in synaptosomes and this may be related to voltage-dependent Ca^{2+} channels involved in neurotransmitter release [29], therefore it may contribute to the inhibitory effect of RuR on electrically evoked release. High intracellular free Ca^{2+} could reduce the calcium influx by reducing the driving force. Therefore, it is conceivable that the inhibition of the Ca_0-dependent evoked release of ACh is due to the reduction of the driving force of Ca. However, the finding that the increase of Ca_0 did not counteract or reduce the effect of CCCP suggests that this explanation can also easily be excluded.

Another possibility is that the decrease in the amount of electrically evoked ACh release caused by these compounds is due to the enhanced resting release of ACh, resulting in an increased biophase concentration of ACh surrounding the axon terminals which in fact inhibits the evoked release of ACh through negative feedback modulation. The enhanced steady-state level of ACh might have been able to reduce the evoked release of ACh via stimulation of presynaptic muscarinic receptors [21, 30]. This explanation can also be excluded since blocking the presynaptic muscarinic receptors by atropine the inhibitory effect of CCCP was not significantly affected.

It has been shown that a rise in intracellular Ca in neurones may also influence a number of physiological processes, including membrane excitability [31, 32], and activation of K-selective channels [33, 34]. The increase in the K^+ permeability of the plasma membrane results in hyperpolarization and a subsequent decrease of the amount of calcium influx at the release site [34]. This mechanism might explain the inhibitory effect of the increase of intracellular Ca^{2+} on the evoked release of ACh. This suggestion is substantiated by the finding that quinine, an inhibitor of K^+ permeability, partly prevented the effect of CCCP (Table 1).

In conclusion the most likely explanation is that RuR and CCCP enhance the concentration of free Ca in the cytoplasm and enhance spontaneous release, but enhancing K^+ permeability which in fact results in a decrease of the amount of calcium influx at the release site and hyperpolarization [34] they reduce the evoked release of ACh.

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