ACTIVATION OF GUANYLATE CYCLASE AS SECONDARY REACTION FOLLOWING INCREASE IN INTRACELLULAR Ca ION CONCENTRATIONS

Issei TAKAYANAGI, Tetsuhiro HISAYAMA and Takeshi KOTSUGAI

Department of Chemical Pharmacology, Toho University School of Pharmaceutical Sciences,
Miyama, Funabashi, Chiba 274, Japan

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It was reported by Schultz et al. (1) that removal of Ca^{2+} from bath fluid failed to increase the tissue level of cyclic GMP produced by smooth muscle stimulants. On the other hand, Wallach and Pastan (2) reported that guanylate cyclase activity in cultured fibroblasts was stimulated by physiological concentrations of Ca^{2+} . These studies suggest the possibility that increase in the tissue level of cyclic GMP induced by smooth muscle stimulants does not initiate smooth muscle contraction but due to activation of guanylate cyclase following the increase of the intracellular Ca^{2+} concentrations. In the present study, we attempted to assess whether or not interactions of the smooth muscle stimulants with their receptors increase cyclic GMP formation, after increasing intracellular Ca^{2+} concentrations to induce contraction of smooth muscle.

Takayanagi et al. (3) found that, though a cholinergic stimulant and serotonin increased the tissue level of cyclic GMP in the guinea pig taenia caecum, the tissue level of cyclic GMP in the longitudinal muscle of rat duodenum was not increased by either of the stimulants, when the concentrations used were sufficient to induce a maximal contraction. Therefore, two kinds of smooth muscle were used in this study. Male Wistar rats weighing 250–350 g were

killed by a blow on the neck and the duodenum was isolated. The longitudinal muscle was dissected free from the circular muscle and mucosa. Male guinea pigs weighing 350–400 g were also killed by a blow on the neck and taenia was isolated from the caecum.

Guanylate cyclase preparations were obtained by a modification of the method of Kimura and Murad (4). The muscle was homogenized with a glass-Teflon homogenizer in 9 volumes of 10 mM Tris (pH 7.4 at 4°C) containing 0.3 M mannitol and 0.2 mM EDTA-2Na. The homogenate was centrifuged at $100,000\times g$ for 60 min. The pellet (a microsomal fraction) and supernatant were used as guanylate cyclase preparations. All the procedures were performed at 0°C. Guanylate cyclase activity in the supernatant also was assayed by a modification of the method of Kimura and Murad (4). Reactions were initiated by adding 330–660 $\mu\text{g}/\text{ml}$ of protein in the medium which consisted of 50 mM PIPES (pH 7.0 at 37°C), 0.2 mM GTP, 0.5 mM MgCl_2 , 10 mM creatine phosphate, 1 mM dithiothreitol, 1.5 mM MIX, 200 $\mu\text{g}/\text{ml}$ creatine kinase and 2 mM GEDTA plus various concentrations of CaCl_2 (Ca-buffer). The reactions were allowed to proceed for 4 min at 37°C. Five volumes of 50 mM acetate buffer (pH 4) were added to

the medium. After exposing the preparation to 95°C for 3 min the mixture was centrifuged at 1,300×g for 15 min and the supernatant was used for estimation of cyclic GMP. Guanylate cyclase activity in the microsomal fraction was then assayed. The reaction medium contained 1 mg MgCl₂ and 2 mg CaCl₂ according to an ion composition of physiological solution used to assess the mechanical activities of the tissues, because Ca and Mg ions play an important role in an interaction of acetylcholine with the receptors (5). Reactions were initiated by adding 67–80 µg/ml of protein to the medium which consisted of 50 mM PIPES, 0.2 mM GTP, 4 mM MnCl₂, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM creatine phosphate, 1 mM dithiothreitol, 1.5 mM MIX and 200 µg/ml creatine kinase. Other procedures were the same as those mentioned in the experiments with the supernatant. The amounts of cyclic GMP formed were estimated by the radioimmunoassay method of Steiner et al. (6). The concentrations of Ca²⁺ were adjusted by varying the ratio of total Ca and GEDTA and were calculated as described by Ogawa (7). Protein was assayed by the method of Lowry et al. (8), using bovine serum albumin as a standard. Significance was determined by means of Student's *t*-test for paired values.

Compounds used: PIPES (piperazine-N,N'-bis(2-ethanesulfonic acid), Wako), EDTA-2Na (ethylenediamine-N,N'-tetraacetate sodium, Wako), GEDTA (glycol-

etherdiamine tetraacetic acid, Wako), GTP (guanosine-5'-triphosphate, Boehringer-Mannheim), dithiothreitol (Boehringer-Mannheim), Tris (tris-(hydroxymethyl) aminomethane, Sigma), 8-³H-cyclic GMP (15 Ci/mmol, Radiochemical Centre), Mix (3-isobutyl-1-methylxanthine, Sigma) and acetylcholine chloride (Dai-ichi).

The microsomal fractions were treated by acetylcholine in concentrations of 10⁻⁶ and 10⁻⁵ M, which were sufficient concentrations to produce the maximal concentration of the guinea pig taenia caecum and longitudinal muscle of rat duodenum.

Acetylcholine (10⁻⁶ and 10⁻⁵ M) did not influence the guanylate cyclase activity in the microsomal fractions derived from the guinea pig taenia caecum and longitudinal muscle of rat duodenum (Table 1). These results suggest that the increase in the tissue level of cyclic GMP in the muscle contracted by the smooth muscle stimulant is not mediated directly through the drug-receptor interaction.

Figure 1 shows the effects of Ca²⁺ concentrations on guanylate cyclase activity in the supernatant obtained from the both smooth muscles. Guanylate cyclase activity in the supernatant obtained from the guinea pig taenia caecum was higher than that in the longitudinal muscle of rat duodenum (Fig. 1). The activity of the soluble guanylate cyclase obtained from the guinea pig taenia caecum was significantly enhanced by Ca²⁺ in concentrations over 10⁻⁶ M, while Ca²⁺ in all

Table 1. Effects of acetylcholine on membrane-bound guanylate cyclase preparations

	Guinea pig taenia caecum	Longitudinal muscle of rat duodenum
Control	410.6±23.5	160.5±14.6
10 ⁻⁶ M ACh	423.8±18.9	167.2± 9.5
10 ⁻⁵ M ACh	436.0±16.2	159.4±13.2

Values (pmoles cyclic GMP formed/min/mg protein) are presented as means±S.E. of 4 experiments. The values in the presence of acetylcholine were not significantly different from the corresponding control values at P<0.05.

Fig. 1. Effects of Ca^{2+} concentrations on soluble guanylate cyclase preparations. Upper: from the guinea pig taenia caecum, lower: from the longitudinal muscle of rat duodenum. Ordinate: guanylate cyclase (GC) activity (pmoles/mg protein), abscissa: Ca^{2+} concentration (M). 2G: in the presence of 2 mM GEDTA. Values are presented as means with S.E. of 4 experiments. The values estimated in the preparation from the guinea pig in the higher concentrations (more than 10^{-6} M) of Ca^{2+} were significantly different from the value in the presence of 2 mM GEDTA (2G) at $P < 0.05$.

cellular Ca^{2+} concentrations (2). The microsomal fraction used in the present work contained the plasma membrane and sarcoplasmic reticulum (9). The fact that application of acetylcholine to the microsomal fractions did not activate guanylate cyclase suggest that the increase of the tissue level of cyclic GMP in the smooth muscle contracted by the stimulants is not mediated directly through the drug receptor but rather is due to a secondary reaction, after the contraction of smooth muscle. Since activation of guanylate cyclase in the supernatant derived from the guinea pig taenia caecum was activated by concentrations over 10^{-6} of Ca^{2+} , which is the intracellular concentrations required to produce contraction of smooth muscle (10), the increase of cyclic GMP brought about in the guinea pig taenia caecum by the stimulant is considered to be due to activation of guanylate cyclase following the increase of intracellular Ca^{2+} concentration. The soluble guanylate cyclase in the longitudinal muscle of rat duodenum was not activated by the concentrations up to 3×10^{-5} M of Ca^{2+} . These observations suggest that the tissue level of cyclic GMP in the longitudinal muscle of rat duodenum was not increased by the smooth muscle stimulants (3). Furthermore, a difference in the effects of Ca^{2+} on two soluble guanylate cyclase preparations suggest that guanylate cyclase in the taenia caecum of guinea pig and that of the longitudinal muscle of rat duodenum have different properties, as already reported by Takayanagi et al. (11).

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