RELATIONSHIP BETWEEN THE LEVELS OF INTRACELLULAR CYCLIC NUCLEOTIDES AND MECHANICAL RESPONSES INDUCED BY DRUGS

Hideo OHKUBO, Issei TAKAYANAGI and Keijiro TAKAGI

Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

Accepted October 13, 1975

Abstract—The tracheal smooth muscle dissected free from connective tissues was used as a test preparation, since isoprenaline caused a decrease in cyclic GMP in the muscle while it caused an increase in cyclic GMP in the remaining tracheal tissue. Acetylcholine, histamine and papaverine increased both cyclic AMP and cyclic GMP levels in the tracheal muscle. Isoprenaline increased the cyclic AMP level but decreased the cyclic GMP level. However, when tracheal muscle was incubated with isoprenaline in the presence of acetylcholine, isoprenaline did not cause a decrease in cyclic GMP but rather a significant increase in cyclic AMP after only 1 min incubation. These results indicate that the relaxation of the tracheal muscle by isoprenaline is initiated by the increase in cyclic AMP but is not associated with the change in cyclic GMP.

Several authors have reported that elevation of the intracellular cyclic AMP level is associated with relaxation of smooth muscle induced by isoprenaline or papaverine (1, 2, 3, 4, 5, 6). It has also been reported that acetylcholine and other muscarinic receptor stimulants increase the intracellular level of cyclic GMP in mammalian heart muscle, brain, lung slices and intestinal smooth muscle (2, 7, 8, 9, 10, 11). Recently, Murad and Kimura (12) tested the effects of several drugs on cyclic AMP and cyclic GMP levels in incubations of guinea pig tracheal ring preparations and concluded that drugs which can relax the tracheal muscle increased the cyclic AMP level and drugs which can contract the muscle increased the cyclic GMP level. It is, however, well known that the tracheal smooth muscle contracted by acetylcholine is relaxed by beta-adrenomimetics. Therefore, the aim of the present work was to test changes in cyclic AMP and cyclic GMP levels involved in antagonism between acetylcholine and isoprenaline.

MATERIALS AND METHODS

Female Hartley guinea pigs (250–350 g in body weight) were sacrificed by a blow on the head and then bled from the femoral artery. The trachea was isolated and placed in a Locke-Ringer solution. The muscular tissue of the trachea was excised carefully from connective tissues and chondrin. Only this smooth muscle was used as a test preparation. The muscle isolated from one animal was divided into 2. Six pieces of the tracheal muscle from 6 animals, the amount of muscle required to estimate simultaneously the contents of cyclic AMP and cyclic GMP was pooled in one incubation tube for the test and 6 pieces of each pair were pooled in another tube for the control.
These were preincubated for 90–120 min in Locke-Ringer solution at 37°C aerated with air. After the exposure to test drugs for various periods, the incubation was terminated using liquid nitrogen. The paired t-test was performed in this experiment. Locke-Ringer solution had the following composition (g/l): NaCl 9.0, KCl 0.4, CaCl₂ 0.2, MgCl₂ 0.2, NaHCO₃ 0.5 and glucose 0.5.

The muscle preparations were homogenized with a glass homogenizer in 2 ml of cold 6% trichloracetic acid which contained 0.5 p moles of [³H]-cyclic AMP to estimate the recovery of cyclic nucleotides. Homogenate was centrifuged at 1000 × g at 0°C for 30 min, and the supernatant was acidified by 1 N HCl, thereafter trichloracetic acid was extracted 4 times with 3 volumes of ether. The cyclic nucleotides samples were lyophylized. The lyophylized samples were dissolved with distilled water and these were applied to Sephadex G-25 (0.9 × 2.4 cm) columns which were washed with 1.2 ml water, and then with cyclic nucleotides, were eluted with 2.5 ml water. The solution of cyclic nucleotides was lyophylized to dryness and redissolved with 200 µl of 50 mM sodium acetate buffer, pH 6.2. A hundred µl of this solution was used for estimation of cyclic GMP, 25 µl for estimation of cyclic AMP and 25 µl for calculating recovery.

Cyclic AMP assay: Cyclic AMP was assayed with the method of Gilman (13). Reactions were initiated by addition of 10 µl of 2.5 × 10⁻⁴ g/ml of protein kinase in the mixtures, 25 µl of cyclic nucleotide sample plus 25 µl of 50 mM sodium acetate buffer, pH 4.0 plus 50 µl of [³H]-cyclic AMP (0.5 p moles) in 50 mM sodium acetate buffer, and were allowed to proceed for 60 min at 0°C. The mixtures diluted in 1 ml of cold 20 mM potassium phosphate buffer, pH 6.0, were passed through a Millipore filter (HAWP-02500), and the filter was washed with 10 ml of this buffer and placed in a counting vial with 1 ml of methyl cellosolve.

Cyclic GMP assay: Cyclic GMP content was assayed with the radioimmunoassay of Steiner et al. (14) modified by Yasuda et al. (15). Reactions were initiated by addition of 50 µl of antiserum of cyclic GMP in the mixtures, which consisted of 100 µl of cyclic nucleotide sample and 50 µl of [³H]-cyclic GMP (1.33 p moles), and allowed to proceed for 90 min at 0°C. The mixtures were passed through a Millipore filter (HAWP-02500), and the filter was washed with 6 ml of cold sodium acetate buffer, pH 6.2, and placed in a counting vial with 1 ml of methyl cellosolve. Twenty five µl of cyclic nucleotides sample was put into a counting vial with 1 ml of methyl cellosolve for the purpose of calculating final recovery.

The radioactivity was counted using a Packard Tri-Carb liquid scintillation counter (model 3203) in 9 ml of toluene scintillator, which contained 4.8 g of DPO, 600 ml of toluene and 300 ml of methyl cellosolve.

The trichloracetic acid precipitate was solubilized with 1 N NaOH and assayed for protein by the method of Lowry et al. (16) using bovin serum albumin as a standard. Mean content of protein of the muscle was 0.74 mg/a preparation from one animal.

Drugs: 8-[³H]-Cyclic AMP (27.5 ci/m mole) and 8-[³H]cyclic GMP (15 ci/m mole) were purchased from Radiochemical Center. Acetylcholine chloride, histamine hydro-
chloride and isoprenaline hydrochloride were obtained from Kaken Kagaku, Tokyo, Japan and papaverine hydrochloride from Daiichi Chemical Co., Tokyo, Japan. Protein kinase of cyclic AMP was purchased from Sigma Chemical Co..

RESULTS

The intracellular cyclic AMP and cyclic GMP contents (mean±S.E.) of the untreated preparation were 21.9±2.0 (N=43) and 0.32±0.04 (N=27) in the tracheal muscle and 4.5±1.1 (N=6) and 0.15±0.01 (N=6) p moles/mg of protein in the residual tissues, respectively.

Isoprenaline (10⁻⁷ g/ml and 10⁻⁶ g/ml), which relaxed the tracheal muscle submaximally or maximally, increased the intracellular cyclic AMP level. Acetylcholine (10⁻⁵ g/ml) and histamine (10⁻⁵ g/ml), which caused a submaximal contraction of the tracheal muscle, also caused an elevation in the cyclic AMP level. However, a further increase in the cyclic AMP level by isoprenaline in the presence of acetylcholine was not observed after 10 min.

| Drugs (g/ml) | min | Cyclic AMP level | p moles/mg protein | relative % | N | p value |
|-------------|-----|------------------|--------------------|------------|---|---------|
| Control     | 10  | 15.8±2.6         | 100                | 8          |   | <0.05   |
| Isoprenaline 10⁻⁷ | 10  | 23.9±2.7         | 199.5±53.0        | 8          |   | <0.05   |
| Control     | 5   | 36.7±4.3         | 100                | 5          |   | <0.05   |
| Isoprenaline 10⁻⁶ | 10  | 65.5±8.5         | 190.9±36.3        | 5          |   |         |
| Control     | 10  | 14.4±2.4         | 100                | 5          |   | <0.01   |
| Acetylcholine 10⁻⁵ | 5   | 31.3±1.6         | 251.9±51.2        | 5          |   | <0.01   |
| Control     | 15  | 10.1±1.4         | 100                | 5          |   |         |
| Histamine 10⁻⁵ | 5   | 37.0±5.4         | 429.4±137.9       | 5          |   | <0.01   |
| Acetylcholine 10⁻⁵ | 15  | 27.7±2.3         | 100                | 7          |   |         |
| Acetylcholine 10⁻⁵ | 15  | 25.8±4.1         | 92.8±10.0         | 7          |   | >0.05   |
| Isoprenaline 10⁻⁷ | 10  | 25.8±4.1         | 92.8±10.0         | 7          |   | >0.05   |
| Acetylcholine 10⁻⁵ | 15  | 38.4±4.1         | 100                | 5          |   |         |
| Acetylcholine 10⁻⁵ | 15  | 44.5±3.2         | 119.4±9.6         | 5          |   | >0.05   |
| Histamine 10⁻⁵ | 15  | 23.1±2.9         | 100                | 5          |   |         |
| Histamine 10⁻⁵ | 15  | 21.6±2.5         | 98.1±15.2         | 5          |   | >0.05   |
| Histamine 10⁻⁵ | 15  | 32.2±2.7         | 100                | 10         |   | >0.05   |
| Histamine 10⁻⁵ | 15  | 36.8±3.3         | 121.1±17.8        | 10         |   | >0.05   |
| Isoprenaline 10⁻⁷ | 10  | 14.6±0.7         | 100                | 6          |   |         |
| Papaverine 3×10⁻⁵ | 10  | 35.9±2.3         | 249.0±23.9        | 6          |   | <0.01   |

Preincubated with acetylcholine or histamine for 5 min, smooth muscle preparation was incubated with isoprenaline for 10 min in the presence of each agonist.

Relative %: The paired t-test was performed and cyclic AMP level was indicated as a percentage of the level in each control (100%).

N: Number of experiments.
incubation, as compared to the level (100%) which had been induced by acetylcholine or histamine alone (Table 1). Acetylcholine and histamine induced a significant increase in the cyclic GMP level after 5 min incubation, while isoprenaline decreased the level in cyclic GMP. A decrease in the cyclic GMP level induced by isoprenaline was not observed in the presence of acetylcholine (Table 2).

In order to determine accurately any change produced by isoprenaline in the levels of cyclic nucleotides, we studied the time course of effects of isoprenaline on the levels of the cyclic nucleotides in the presence and absence of acetylcholine. Fig. 1 shows that acetylcholine produced a rapid accumulation of cyclic GMP which reached a maximum at 2 min and then slowly declined. Isoprenaline produced a rapid cyclic AMP increase and cyclic GMP decrease which also reached a maximum at 2 min. After the 5 min incubation with acetylcholine, isoprenaline induced a slight increase in cyclic AMP and decrease in cyclic GMP level in the presence of acetylcholine. However, the changes in cyclic nucleotide levels in the time course study were minute and insignificant, since each point in Fig. 1 is represented as a percent content to zero time. Further investigation was carried out to determine more precisely the effects of isoprenaline on the intracellular levels of the cyclic AMP and cyclic GMP in the presence of acetylcholine. Cyclic AMP and cyclic GMP were estimated as a percentage of the levels against the cyclic nucleotide levels induced by acetylcholine alone after 1, 2 and 10 min incubations of the tracheal muscle with isoprenaline in the presence of acetylcholine. Isoprenaline induced an increase in cyclic AMP but not a decrease in cyclic GMP after 1 min incubation in the presence of acetylcholine. Isoprenaline had no effect on the levels of either cyclic nucleotide when the tracheal smooth muscle preparations were incubated for more than 2 min (Table 3). Papaverine (3 × 10⁻⁵

| Drugs (g/ml) | min | Cyclic GMP level |
|-------------|-----|------------------|
|             |     | p moles/mg protein | relative % | N   | p value |
| Control     | 5   | 0.31±0.08        | 100        | 6   | <0.01   |
| Acetylcholine 10⁻⁵ | 5   | 1.10±0.16        | 532.8±171.7 | 6   | <0.05   |
| Control     | 5   | 0.18±0.04        | 100        | 6   |         |
| Histamine 10⁻⁵ | 5   | 0.63±0.26        | 317.9±71.9  | 6   | <0.05   |
| Control     | 10  | 0.47±0.17        | 100        | 5   |         |
| Isoprenaline 10⁻⁸ | 10  | 0.39±0.17        | 74.4±7.8   | 5   | <0.05   |
| Acetylcholine 10⁻⁵ | 15  | 0.66±0.20        | 100        | 8   |         |
| Acetylcholine 10⁻⁸ | 15  | 0.66±0.18        | 134.4±37.4 | 8   | >0.05   |
| + Isoprenaline 10⁻⁸ | 15  | 0.56±0.11        | 115.5±23.8 | 6   | >0.05   |
| Histamine 10⁻⁵ | 15  | 0.54±0.08        | 198.2±41.5 | 4   |         |
| Control     | 10  | 0.22±0.06        | 100        | 4   |         |
| Papaverine 3 × 10⁻⁵ | 10  | 0.39±0.08        | 198.2±41.5 | 4   | >0.05   |

Incubations were carried out as described in Table 1.
g/ml), a typical smooth muscle relaxant, caused a greater increase in the cyclic AMP level than in the cyclic GMP level.

Acetylcholine also increased both cyclic nucleotide levels in the remaining tracheal tissue. As mentioned above, isoprenaline caused an increase in cyclic AMP and a decrease in cyclic GMP in the smooth muscle preparation, whereas in the remaining tracheal tissue it caused an increase in cyclic GMP as shown in Fig. 2.

---

**Fig. 1.** Time course of the effects of acetylcholine and isoprenaline on cyclic AMP and cyclic GMP levels in guinea pig tracheal smooth muscle.

(●): Acetylcholine 10^{-5} g/ml alone (1–5 min)
Acetylcholine 10^{-5} g/ml + isoprenaline 10^{-6} g/ml (5–15 min)
(○): Isoprenaline 10^{-6} g/ml alone

Solid line: Cyclic GMP level, Broken line: Cyclic AMP level. Each point represents mean±S.E. of 4–8 experiments. Ordinate: Cyclic nucleotide levels (%) in logarithm scale. Abscissa: Time (min) after application of the drugs.

---

**Fig. 2.** Time course of the effects of acetylcholine and isoprenaline on cyclic nucleotide levels in the remaining tracheal tissue.

(●): Acetylcholine 10^{-5} g/ml (○): Isoprenaline 10^{-6} g/ml

Solid line: Cyclic GMP level, Broken line: Cyclic AMP level. Each point represents mean±S.E. of 2–4 experiments. Ordinate: Cyclic nucleotide levels (%) in logarithm scale. Abscissa: Time (min) after application of the drugs. Significantly different from the zero time control; *p<0.05, **p<0.01.
DISCUSSION

It was clearly demonstrated herein that application of isoprenaline alone increases the cyclic AMP level and decreases the cyclic GMP level and that papaverine, which is a potent phosphodiesterase inhibitor (17, 18, 19), induces a greater increase in the cyclic AMP level than in the cyclic GMP level and also that acetylcholine produces a greater and more rapid increase in the cyclic GMP level than in the cyclic AMP level. These results support the hypothesis that the increase of the intracellular cyclic AMP level is associated with the relaxation of the smooth muscle and the increase of the intracellular level of cyclic GMP with the contraction. It is also considered from these data that the responses of the tracheal muscle to isoprenaline, papaverine and acetylcholine are determined by a change of the cyclic AMP/cyclic GMP ratio.

It is well known that the contractions produced by acetylcholine and histamine are inhibited by isoprenaline. In this study, the cyclic AMP and cyclic GMP levels were not affected by a 10 min incubation of the tracheal muscle with isoprenaline in the presence of acetylcholine and histamine. In this experimental condition, the contraction of the tracheal muscle produced by acetylcholine or histamine relaxed completely after application of isoprenaline. These results suggest that the relationship between cyclic AMP and cyclic GMP levels in these antagonisms is not simple. The experimental result (Table 3) indicated that cyclic AMP level in the tracheal muscle significantly increased after only one min incubation with isoprenaline in the presence of acetylcholine. This observation suggests that the relaxation of the acetylcholine-induced contraction by isoprenaline is initiated by

### Table 3. Effects of isoprenaline on cyclic nucleotide levels in the presence of acetylcholine

| Drugs (g/ml) | min | Cyclic AMP | Cyclic GMP | N |
|-------------|-----|------------|------------|---|
| Control     |     | 100        | 100        | 5 |
| Isoprenaline 10⁻⁶ | 1   | 199.3±18.5** | 75.1±6.9*  | 5 |
| Control     |     | 100        | 100        | 5 |
| Isoprenaline 10⁻⁶ | 10  | 190.9±36.3* | 74.4±7.8*  | 5 |
| Control     |     | 100        | —          | 10|
| Acetylcholine 10⁻⁷ | 1   | 132.7±14.0* | —          | 10|
| Acetylcholine 10⁻⁷ | 6   | 100        | 100        | 6 |
| Acetylcholine 10⁻⁵ | 6   | 100        | 100        | 6 |
| +Isoprenaline 10⁻⁵ | 1   | 140.8±12.6* | 102.9±15.0 | 6 |
| Acetylcholine 10⁻⁵ | 7   | 100        | —          | 5 |
| Acetylcholine 10⁻⁵ | 7   | 129.3±23.0  | —          | 5 |
| +Isoprenaline 10⁻⁵ | 15  | 100        | 100        | 5 |
| Acetylcholine 10⁻⁵ | 15  | 119.4±9.6  | 128.3±30.5 | 5 |

The preparations were preincubated with acetylcholine for 5 min and isoprenaline was added at the various times indicated.

*: p<0.05,  **: p<0.01
the rapid increase of the intracellular cyclic AMP level and is not concerned with the intracellular level of cyclic GMP.

Our data which show that acetylcholine and histamine increase both cyclic GMP and cyclic AMP levels are similar to those of Murad and Kimura (12) who stated that the contractions of the tracheal ring preparation produced by acetylcholine and histamine are associated with the cyclic GMP level, since the increase in the cyclic GMP level produced by both drugs was blocked by their corresponding competitive antagonists or atropine and diphenhydramine and that the increase of cyclic AMP was blocked by beta-adrenergic blockers.

Acknowledgements: The authors are sincerely grateful to Prof. M. Yamada and Dr. H. Yasuda, Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, for providing the antisera of cyclic GMP.

REFERENCES

1) Andersson, R.: Acta. physiol. scand. Suppl. 382, 1 (1972)
2) Lee, T.P., Kuo, J.F. and Greengard, P.: Proc. natn. Acad. Sci. 69, 3287 (1972)
3) Takayanagi, I., Uchida, M., Inatomi, N., Tomiyama, A. and Takagi, K.: Japan. J. Pharmacol. 22, 869 (1972)
4) Inatomi, N., Takayanagi, I., Uchida, M. and Takagi, K.: Europ. J. Pharmacol. 26, 73 (1974)
5) Inamasu, M., Shinjo, A., Iwasawa, Y. and Morita, T.: Biochem. Pharmacol. 23, 3213 (1974)
6) Inatomi, N., Takayanagi, I. and Takagi, K.: Japan. J. Pharmacol. 25, 63 (1975)
7) George, W.J., Polson, J.B., O'Tool, A.J. and Goldberg, N.D.: Proc. natn. Acad. Sci. 66, 398 (1970)
8) Murad, F., Manganiello, V.C. and Vaughan, M.: Proc. natn. Acad. Sci. 68, 736 (1971)
9) Kuo, J.F., Lee, T.P., Ryes, P.L., Walton, K.G. and Greengard, P.: J. biol. Chem. 247, 16 (1972)
10) Kuo, J.F. and Kuo, W.N.: Biochem. biophys. Res. Commun. 55, 660 (1973)
11) Stoner, J., Manganiello, V.C. and Vaughan, M.: Mol. Pharmacol. 10, 155 (1974)
12) Murad, F. and Kimura, H.: Biochim. Biophys. Acta 343, 275 (1974)
13) Gilman, A.G.: Proc. natn. Acad. Sci. U.S.A. 69, 305 (1970)
14) Steiner, A.L., Parker, C.W. and Kipnis, D.M.: J. biol. Chem. 247, 1106 (1972)
15) Yasuda, H., Yamada, M. and Kurata, S.: Seikagaku 46, 618 (1974) (in Japanese)
16) Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: J. biol. Chem. 193, 265 (1951)
17) Miyamoto, M., Takayanagi, I., Ohkubo, H. and Takagi, K.: Japan. J. Pharmacol. (in press)
18) Lugnier, C. and Stoclet, J.C.: Biochem. Pharmacol. 23, 3071 (1974)
19) Amer, M.S., McKinney, G.R. and Akcasu, A.: Biochem. Pharmacol. 23, 3085 (1974)