Positive Inotropic and Chronotropic Effects of 8-Substituted Derivatives of Cyclic AMP and Activation of Protein Kinase A

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ABSTRACT—Inotropic and chronotropic effects of 8-substituted derivatives of cyclic AMP (8-SH, 8-SCH₂C₆H₅, 8-N₃, 8-SCH₃, 8-Br, 8-N(CH₃)₂, 8-OCH₃) were studied using guinea pig atrial and ventricular muscle preparations and correlated with the activation of the protein kinase A derived from the bovine myocardium. All the compounds produced positive inotropic and chronotropic effects. A good correlation was found between the chronotropic effect and the activation of the enzyme, while such a good correlation was not found between the enzyme activation and the positive inotropic effect. However, after treatment of the preparation with theophylline, the positive inotropic effects of some derivatives were potentiated to such a degree that the positive inotropic effects became well-correlated to the activation of the protein kinase. To elucidate the mechanism of the potentiation by theophylline, the effects of 8-phenyltheophylline and 3-isobutyl-1-methylxanthine on the positive inotropic effects of 8-Br and 8-OCH₃ cyclic AMPs were studied. While 3-isobutyl-1-methylxanthine potentiated the effects of both compounds, 8-phenyltheophylline potentiated the effect of only 8-OCH₃ cyclic AMP and only in the atria. These results suggest that the positive inotropic and chronotropic effects of 8-substituted cyclic AMP essentially due to the activation of the protein kinase A, with the hydrolysis of the compounds by phosphodiesterase and (in the atria) activation of adenosine R-receptor subserving the negative inotropic effect intervening.

Cyclic adenosine 3',5'-monophosphate (cyclic AMP) plays an important role as a second messenger of the positive inotropic and chronotropic effects induced through stimulation of cardiac β-adrenergic receptors by catecholamines (1, 2). However, cyclic AMP itself when administered from outside of the cells does not produce positive inotropic and chronotropic effects because the rate of entry of this material into the myocardial cells is slow compared with the rate of degradation by phosphodiesterase due to its low penetrability of the cell membrane (1, 3, 4). In contrast, derivatives of cyclic AMP having lipophylic side chains such as dibutyryl cyclic AMP (5, 6) and benzyl thiobutyl cyclic AMP (7, 8) have been found to produce positive inotropic and chronotropic effects in isolated cardiac muscle preparations. We have studied inotropic and chronotropic effects of derivatives of cyclic AMP in guinea pig atrial preparations and found that 8-substituted derivatives of cyclic AMP (8-R cAMPs) produced the positive inotropic and chronotropic effects, while 2-substituted derivatives (2-R cAMPs) produced the negative ones (9, 10). In the present study, an
attempt was made to define the importance of the activation of protein kinase A for the positive inotropic and chronotropic effects of these derivatives. It is known that 8-R cAMPs are the activators of protein kinase A (11–13).

MATERIALS AND METHODS

Determination of protein kinase A activity

The assay of protein kinase activity based on the phosphorylation of histone was carried out essentially as described by Miyamoto et al. (14), and the experimental conditions were similar to those described previously (15). Briefly, the activation of the bovine myocardial protein kinase A was assessed in a 0.1-ml incubation mixture containing 50 μmoles HEPES (pH 7.0), 5 μmoles MgCl₂, 10 μg histone, 0.1 nmoles γ-32P-ATP, 2 μg protein kinase enzyme and various concentrations (5 × 10⁻⁴–2.5 × 10⁻⁵M) of the cyclic AMP or 8-R cAMPs. After a suitable incubation time to give kinetically valid data, 2 ml of 10% Cl₃CCO₂H was added to the incubation mixture and kept for 10 min at 4°C. The mixture was then filtered under reduced pressure through Whatman glass-fiber filters (GF/C) using Automatic Cell Harvester Lavomash (LH-101, Labo Science, Tokyo, Japan), and the filters were washed with 3 ml of 5% Cl₃CCO₂H. The amount of 32P covalently bound to histone was determined by counting the radioactivity remaining on the filters with a liquid scintillation spectrophotometer using 5 ml of a Triton-toluene based scintillation fluid. The cyclic AMP derivatives being tested as an activator were assayed at no less than seven different concentrations in duplicate. The data thus obtained were analyzed by Lineweaver-Burk plots, and the apparent Ka was determined from the X intercept (−1/Ka) as shown in Fig. 1.

Examination of inotropic and chronotropic effects of 8-R cAMPs

Male albino guinea pigs weighing between

![Graph](attachment:Graph.png)

**Fig. 1.** Effects of cyclic AMP concentration on the activity of cyclic AMP-dependent protein kinase. Incubation conditions were as described in the text.
320–680 g were stunned by a blow on the head. The hearts were rapidly removed, and the right and left atria and the papillary muscle of the right ventricle were dissected in cold bathing solution. The preparations were suspended individually in 8-ml organ baths for recording isometric contractions. The bathing solution was a Krebs-Henseleit’s solution (32 ± 0.1°C) consisting of 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 25 mM NaHCO₃, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, and 11 mM glucose, which was continuously bubbled with 95% O₂ + 5% CO₂. The initial tensions of 0.5 g and 0.25 g were applied to the atrial preparations and papillary muscle preparations, respectively. After 30 min, the optimal resting tension was determined and maintained thereafter. The right atrium was allowed to beat spontaneously, and the left atrium and papillary muscle were stimulated by square pulses at a frequency of 1 Hz, 1 msec duration and voltages of about 50% above the threshold supplied by a square-wave pulse stimulator (Nihon Kohden MSE-3) via a pair of the silver plate electrodes between which the preparations were placed. The isometric contraction was measured by a force-displacement transducer (Toyo Baldwin T7-30-240) connected to a carrier-amplifier (Nihon Kohden RP-5), and the heart rate was counted by a cardiotachometer (Nihon Kohden RT-5). All the measurements were recorded on a thermostylus recorder (Watanabe Sokki Linear Corder Mark V). An equilibration period of 60 min was allowed before starting the experiments. Because of the low solubility of 8-R cAMPs, they were dissolved in Krebs-Henseleit’s solution and applied to the preparation by replacing less than 1.2 ml of the bathing solution. The positive inotropic and chronotropic effects of 8-R cAMPs were expressed as % of the maximum response evoked by 10⁻⁷ M isoproterenol before the administration of test compounds in each preparation.

Materials

The following chemicals were used: histone (Worthington HLY), γ-³²-ATP (7.0 Ci/mmol, New England Nuclear), (-)-isoproterenol (Nikken Kagaku Co.), theophylline ethylenediamine (aminophylline, Eisai Co.), 8-phenylthiotheophylline (Sigma Chemicals) and 3-isobutyl-1-methylxanthine (Sigma Chemicals). Bovine myocardial protein kinase A was purchased from Sigma Chemicals as a crude lyophilized powder. 8-R cAMPs (8-Br, 8-SH, 8-SCH₃, 8-SCH₂C₆H₅, 8-OCH₃, 8-N₃, 8-N(CH₃)₂) were kindly supplied as the free acid by the Seishin Pharmaceutical Co.

Analysis

Statistical significance was estimated using the unpaired Student’s t-test, and linear regression analysis was also conducted. P values of less than 0.05 were considered to be significant.

RESULTS

Protein kinase A activation

In Table 1, the potency of activation of bovine myocardial protein kinase A by 8-R cAMPs is expressed with Ka’ values (for the definition of Ka’, see Table 1). Five compounds (8-SH, 8-SCH₂C₆H₅, 8-N₃, 8-Br, 8-SCH₃) were found to be more potent than cyclic AMP itself, while the other two compounds (8-OCH₃, 8-N(CH₃)₂) were less potent. The order of potency was in agreement with the results of previous studies conducted with bovine brain enzyme (11, 12).

Positive inotropic and chronotropic effects of 8-R cAMPs

Cumulative applications of 8-R cAMPs produced the concentration-dependent positive chronotropic and inotropic effects in guinea pig right and left atrial preparations and papillary muscle preparations in concentrations of 3 × 10⁻⁵ to 3 × 10⁻³ M. Because of the low solubility of the compounds, higher concentrations were not tested. For each compound, the positive inotropic effects on left atrial and papillary muscle preparations evolved slowly, reaching a steady maximal level (within 40 min
| R (8-substituent) | Protein kinase activation (Ka') | 30% of ISP max (× 10⁻⁴ M) | after theophylline 2 × 10⁻⁴ M |
|------------------|--------------------------------|---------------------------|-----------------------------|
|                  |                                | (A) (B) (C)               | (A) (B) (C)                 |
| SH               | 2.35 ± 0.73                    | 1.70 ± 0.09               | 4.33 ± 0.27                 | 3.08 ± 1.12               | 1.55 ± 0.15               | 1.88 ± 0.17               | 3.08 ± 0.28               |
| SCH₂C₅H₃        | 1.86 ± 0.85                    | 1.88 ± 0.21               | 4.70 ± 0.37                 | 3.03 ± 0.35               | 2.91 ± 0.80               | 2.54 ± 0.39               | 2.18 ± 0.44               |
| N₃              | 1.86 ± 0.36                    | 1.56 ± 0.55               | 14.60 ± 4.32                | 2.63 ± 0.83               | 1.74 ± 0.42               | 3.60 ± 1.90               | 2.85 ± 0.24               |
| Br               | 1.63 ± 0.24                    | 2.93 ± 0.77               | 13.45 ± 3.95                | 12.63 ± 1.92              | 1.91 ± 0.08               | 2.39 ± 1.15               | 2.03 ± 0.59               |
| SCH₃            | 1.26 ± 0.20                    | 1.69 ± 0.20               | 11.08 ± 1.88                | 10.22 ± 1.49              | 1.38 ± 1.48               | 2.41 ± 0.73               | 2.80 ± 0.36               |
| OCH₃            | 0.51 ± 0.13                    | 4.10 ± 1.15               | 28.75 ± 4.52                | 20.75 ± 2.74              | 2.98 ± 0.44               | 7.98 ± 1.75               | 5.98 ± 0.78               |
| N(CH₃)₂         | 0.32 ± 0.17                    | 4.85 ± 0.38               | 22.25 ± 5.41                | 13.95 ± 4.96              | 5.45 ± 0.92               | 12.33 ± 3.24              | 10.91 ± 2.46              |

(A) positive chronotropic effect in the right atrium, (B) positive inotropic effect in the left atrium, (C) positive inotropic effect in papillary muscle. Each value represents the mean ± S.E. (protein kinase activation, n = 4–9; inotropic and chronotropic effects, n = 4). The potency of the positive inotropic and chronotropic effects of each compound is calculated using the concentration of the compound required to produce the response of 30% of ISP max (EC₃₀; see in the text). ISP max: the maximal response evoked by 10⁻⁷ M isoproterenol. Ka' = Ka (cyclic AMP) / Ka (8-R cAMP). Ka for cyclic AMP is 12.5 ± 7.6 nM (n = 16).
in each concentration), while the positive chronotropic effects developed faster than of
the inotropic effects attaining a maximal level (within 20 min in each concentration). Figure
2 shows a typical record obtained with 8-N3 cyclic AMP. The positive inotropic and chro-
notropic effects of 8-N3, 8-Br and 8-SCH2C6H5 cyclic AMP developed faster than
those of the other 8-R cAMPs. Figure 3 (A–C) depict the concentration-effect relation of
the positive chronotropic effects in the right atrium and those of the positive inotropic
effects in the left atrium and papillary muscle produced by 8-R cAMPs. The maximal posi-
tive chronotropic effects produced by these compounds was 50% of the maximal response
evoked by isoproterenol, and the concentra-
tion-response curves were approximately
parallel to each other. However, the positive inotropic effects of some derivatives (8-
N(CH3)2, 8-OCH3, 8-N3) in the left atrial
preparation did not attain to 50% of the max-
imal response by isoproterenol even at the
concentration of 3 × 10^{-3} M. Therefore, the
comparison of the potency of the positive ino-
tropic or chronotropic effects of each compo-
und was conducted using the concentration
of the compound required to produce 30% of
the maximal response (EC30). When the EC30
values thus obtained are plotted against the
Ka’ values for the activation of protein
kinase, a good correlation was found between
the EC30 values for the positive chronotropic
effects and the Ka’ values (Fig. 4A), while no
such good correlations were observed between
the EC30 values for the positive inotropic
effects on the left atrial (Fig. 4B) and papil-
lary muscle preparations and the Ka’ values
(Fig. 4C). All the data used for Fig. 4 are
summarized in Table 1.

**Pretreatment with theophylline**

Our previous studies (9, 10) demonstrated
that the positive inotropic effects of 8-R
cAMPs were potentiated, and the negative ino-
tropic effect of 2-R cAMPs were depressed by
the pretreatment with 2 × 10^{-4} M theophyl-
line. In the present study, further quantita-
tive studies were conducted constructing the
concentration-effect curves of 8-R cAMPs in

![Fig. 2. Typical experiments showing the time course of development of the positive inotropic and chronotropic effects of 8-N3 cyclic AMP in guinea pig right atrial, left atrial and papillary muscle preparations.](image-url)
Fig. 3. Concentration-response relationship of 8-R cAMPs in guinea pig right atrial (A), left atrial (B) and papillary muscle (C) preparations. The data represent the mean of 4 experiments. ISP max: the maximal response evoked by 10^{-7} M isoproterenol. Horizontal broken line is 30% of ISP max (see the legend of Table 1 for details).

Fig. 4. Relationship between positive inotropic and chronotropic effects and the protein kinase activation in the absence (•) and presence of 2 x 10^{-4} M theophylline (○) in guinea pig right atrial (A), left atrial (B) and papillary muscle (C) preparations. Each circle represents the mean value (n = 4) and standard errors of each circle are shown in Table 1. ISP max: the maximal response evoked by 10^{-7} M isoproterenol. A regression line is drawn for the data obtained after the treatment with theophylline.
the presence and absence of theophylline. In Table 1 are listed the EC_{50} values for the positive inotropic and chronotropic effects obtained after treatment of the preparation with theophylline. It was found that theophylline produced a left-ward shift of the concentration-effect curves for the positive inotropic effects of some derivatives (8-N(CH$_3$)$_2$, 8-OCH$_3$, 8-SCH$_3$, 8-Br), while the positive chronotropic effects of 8-R cAMPS were scarcely affected. The potentiation of the positive inotropic effect was more

![Graphs showing changes in positive inotropic effects of 8-Br cyclic AMP and 8-OCH$_3$ cyclic AMP after treatment with 8-phenyltheophylline (8-PT) and 3-isobutyl-1-methyxanthine (IBMX) in guinea pig papillary muscle (A) and left atrial (B) preparations. The data represent the mean ± S.E. (n = 3–4). ISP max: the maximum response evoked by 10^{-7} M isoproterenol. Significant difference from the data obtained with 8-R cAMP alone: *P < 0.05, **P < 0.01. ○: 8-R cAMP; ●: + 8-PT, 3 × 10^{-5} M; ▲: + IBMX, 10^{-5} M.](image-url)
remarkable in the left atria than in the papillary muscle. When EC$_{30}$ values obtained for the positive inotropic effect after theophylline were plotted against ka$^+$ values, good correlations were found (Fig. 4, A–C).

Pretreatment with 8-phenyltheophylline or 3-isobutyl-1-methylxanthine

The potentiation of the positive inotropic effect induced by theophylline involves both the inhibition of adenosine receptors (R-type receptors) and that of phosphodiesterase. In order to elucidate which mechanism is responsible for the potentiation, the effects of 8-phenyltheophylline, a specific antagonist of the R-receptor (16), and 3-isobutyl-1-methylxanthine, a strong inhibitor of phosphodiesterase (17), on the positive inotropic effects of 8-OCH$_3$ and 8-Br cyclic AMPs were investigated. These two compounds were selected as representative compounds, because the positive inotropic effects of these two compounds were strongly potentiated by theophylline. In papillary muscles, the positive inotropic effects of 8-OCH$_3$ and 8-Br cyclic AMPs were potentiated by 10$^{-5}$ M 3-isobutyl-1-methylxanthine, while they were not affected by 3 X 10$^{-5}$ M 8-phenyltheophylline (Fig. 5A). In left atria, 3-isobutyl-1-methylxanthine similarly potentiated the response. On the other hand, 8-phenyltheophylline also potentiated the effect of 8-OCH$_3$ cyclic AMP, whereas the effect of 8-Br cyclic AMP was not affected (Fig. 5B).

DISCUSSION

In the present study conducted in the guinea pig atrial and ventricular muscle preparations, all the 8-R cAMPs used produced concentration-dependent positive inotropic and chronotropic effects; and at the same time, they were found to activate the bovine myocardial protein kinase A. The order of potency of these compounds to elicit the positive inotropic and chronotropic effects was essentially the same as that of the efficacy of these derivatives by a single administration (0.5 mg/l) in the previous study (9). The order of potency for activation of bovine myocardial protein kinase A agreed well with that of the previous studies obtained from bovine brain (11, 12), although these two enzymes may possess different regulatory subunits from each other (18). It has been shown that the activation of a protein kinase by cyclic AMP is responsible for the increased availability of the voltage-dependent calcium channels (1, 19). Moreover, injection of the catalytic subunit of protein kinase A into a single cell increased the slow inward current (20, 21). It is generally accepted that the activation of the slow inward current mediated by cyclic AMP closely correlates to the positive inotropic effect on the heart (1, 2), and Noma and co-workers (22) suggested the key role of the increased slow inward current in the positive chronotropic effect in S-A node cells. A good correlation was found between the positive chronotropic effect and the activation of the protein kinase by 8-R cAMPs, indicating that acceleration of the atrial rate induced by these compounds may depend on their effects on the protein kinase and resultant increase in slow inward current. The positive inotropic effects of 8-R cAMPs on the left atrium and on the right ventricular papillary muscle were not correlated with the potency to activate the bovine myocardial protein kinase. However, in the presence of theophylline, the positive inotropic effects of 8-R cAMPs in guinea pig atrial and ventricular muscle preparations were potentiated, and a good correlation was found between the protein kinase activation and the positive inotropic effects. This indicates that the positive inotropic effects of 8-R cAMPs were fundamentally dependent on the activation of protein kinase, but the inotropic effects of these compounds are modified by additional mechanisms in the intact myocardial cells. Positive chronotropic effects were not affected by theophylline. As it was reported that the positive inotropic effects of cyclic AMP derivatives were potentiated in the presence of 3-isobutyl-1-methylxanthine (23, 24), the effects of 8-phenyltheophylline and 3-isobutyl-1-methylxanthine treatments were studied.
The positive inotropic effects of 8-Br cyclic AMP and 8-OCH₃ cyclic AMP were potentiated by 3-isobutyl-1-methylxantine in atrial and ventricular muscle preparations, suggesting that the hydrolysis of these compounds by phosphodiesterase attenuated the positive inotropic effects of 8-R cAMPs. This interpretation, however, disagrees with the previous biochemical data (11, 12) showing that these compounds are not susceptible to the hydrolysis by the cyclic AMP phosphodiesterase. Furthermore, the activity of cyclic AMP phosphodiesterase has been shown to be almost similar in guinea pig atrial and ventricular myocardium (25).

The positive inotropic effect of 8-OCH₃ cyclic AMP on the left atria was also potentiated by 8-phenyltheophylline, while the positive inotropic effect on papillary muscles was not potentiated. The adenosine R-receptor subserving the negative inotropic and chronotropic effects is present in the atrial preparation (26, 27), but not in ventricular muscle preparations (28, 29). Thus, it is inferred that the R-receptor mediated depressant effect plays a role to attenuate the positive inotropic effect of this compound in the left atria. In this possibility, the present results that the positive inotropic effects of 8-N₃, 8-SH and 8-SCH₂C₆H₅ cyclic AMPs in the ventricle were less affected by the pretreatment with theophylline in comparison with the effects in the left atria may also suggest the importance of the R-receptor.

The reason why the positive chronotropic effects of 8-R cAMPs were not affected by the pretreatment with theophylline is not clear at present. There are two possibilities: 1) the activity of cyclic AMP phosphodiesterase may be lower in pacemaker cells than in ordinary myocardial cells and/or the intracellular level of these derivatives can be preserved against the hydrolysis due to phosphodiesterase in pacemaker cells and 2) the depressent effect of 8-R cAMPs on pacemaker activity through activation of adenosine R-receptor may be minimal. Findings of Tamura et al. (30) that the activity of cyclic AMP phosphodiesterase in specialized myocardial cells of the conduction system was lower than that in ordinary myocardial cells support the first possibility. Furthermore, the membrane permeability of these derivatives in the right atria seems to be higher than that in the left atria and papillary muscles since the positive chronotropic effect was produced at lower concentrations than those required to produce the positive inotropic effects of the same magnitude and a shorter time was required to attain the steady level in comparison with the inotropic effect. Previous workers (for references, see (1)) suggested that the balance between the rate of entry into cells and the rate of hydrolysis by the phosphodiesterase may determine whether cyclic AMP and its analogs produce positive or negative effects. There is also some evidence to support the second possibility. Numerous adenyl compounds as well as cyclic AMP itself produce a decrease in contraction and heart rate via activation of adenosine R-receptors in the atrial preparations (3, 27, 28). The concentration-response curve for the depressant effect on the heart rate of most adenosine analogs is characterized by a steep gradient (31, 32), and the negative chronotropic effect of cyclic AMP is induced only with high concentrations (3, 4).

Recently, the existence of a family of phosphodiesterase isoenzymes has been recognized in various mammalian myocardiums and selective inhibitors of phosphodiesterase III have been extensively examined for their therapeutic efficacies as positive inotropic agents (33). Moos and co-workers (34) have shown that inhibitors of this isoenzyme have some structural similarity to the natural substrate cyclic AMP. Meyer and co-workers (25) reported the inhibitory effects of adenosine and its analogs on cyclic AMP phosphodiesterase in guinea pig atrial and ventricular preparations. However, it has been claimed that 8-R cAMPs do not act as phosphodiesterase inhibitors in guinea pig papillary muscles (23). Endoh and Nakamura (24) have also shown that 8-SCH₂C₆H₅ cyclic AMP does not produce increases in intracellular cyclic AMP, although this compound shows comparatively stronger
potency with respect to the positive inotropic effects among cyclic AMP derivatives. Therefore, it may be concluded that the positive inotropic and chronotropic effects of 8-R cAMPs on the guinea pig heart are essentially due to the activation of protein kinase A. However, the actual magnitude of the positive inotropic effect depends likewise on the variable degree of hydrolysis of these compounds by cyclic AMP phosphodiesterase and, additionally in the atria, on the negative inotropic effect mediated by adenosine R-receptors.

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