Potentialiation by Higenamine of the Aconitine-Induced Positive Chronotropic Effect in Isolated Right Atria of Mice: The Effects of Cholera Toxin, Forskolin and Pertussis Toxin

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Aconitine and higenamine are the major cardioactive compounds obtained from processed aconite. The chronotropic interaction between these two compounds was investigated in isolated right atria of mice. Both aconitine and higenamine potentiated the action of the other. Practolol (1 nM), a selective β_1 -adrenergic antagonist, but not butoxamine (1 μ M), a β_2 -adrenergic antagonist, blocked the potentiation by higenamine (5 nM) of the aconitine-induced positive chronotropic effect and, at high concentrations (30 and 300 nM) also shifted the aconitine concentration-response curves to the right. The potentiating interaction between aconitine and higenamine was reversed by pretreating with cholera toxin (CTX) and forskolin. In CTX (100 nM, 1 h)- and forskolin (30 and 100 nM)-treated atria, higenamine significantly depressed the aconitine-induced response, which was abolished by pertussis toxin (PTX, 150 μ g/kg, i.p., 3 d). Neither CTX (50 and 100 nM) nor forskolin (15–100 nM) significantly affected the aconitine-induced positive chronotropic effect, while PTX (150 μ g/kg) depressed it. These results suggest that the potentiating interaction between aconitine and higenamine involves “cross-talk” between the β_1 -adrenergic signalling pathway and G_i -protein.

Key words aconitine–higenamine interaction; positive chronotropy; cholera toxin; pertussis toxin; mouse right atria

Processed aconite possesses a wide range of pharmacological actions.¹⁾ Aconitine, the main active and toxic compound obtained from aconite root^{2,3)} is well known for its tachyarrhythmic effect in the heart, both *in vivo*^{4–6)} and *in vitro*.^{7,8)} The arrhythmogenic action of aconitine results from direct activation of the sodium channel.⁹⁾ This voltage-dependent sodium channel activation, followed by acetylcholine (ACh) release, triggers muscarinic cholinergic mechanisms which are also involved in aconitine-induced arrhythmia.^{6,8,10)} Higenamine, another cardioactive component of aconite elicits positive chronotropic and inotropic effects by activation of β_1 -adrenergic receptors¹⁰⁾ and Ca^{2+} influx.^{11,12)}

Despite its high toxicity, processed aconite is frequently prescribed in Japanese–Sino medicine for a variety of ailments.¹⁾ In view of the importance of the possible pharmacological interactions between ingredients in the overall activity of the crude aconite preparation, we have been investigating the interaction between aconitine and higenamine in terms of their cardiac actions. Previously, we have reported that higenamine enhances the aconitine-induced positive chronotropic effect in isolated right atria of mice through activation of β_1 -adrenergic receptors,¹⁰⁾ while it antagonizes the aconitine-induced bradycardia *in vivo*.¹³⁾

The aim of the present study was to clarify the mechanism of the chronotropic interaction between aconitine and higenamine, contained in aconite extracts, in isolated right atria of mice by using cholera toxin (CTX), a G_s -protein activator, pertussis toxin (PTX), a G_i -protein inhibitor, and forskolin, a direct activator of adenylyl cyclase.

MATERIALS AND METHODS

Preparation of Isolated Right Atria Male ddY mice

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weighing 30–40 g (7- to 9-weeks old) were sacrificed by decapitation and exsanguination. The beating heart was rapidly removed from the thorax and placed in Krebs–Henseleit solution. The solution was composed of 118.4 mM NaCl, 4.69 mM KCl, 2.0 mM $CaCl_2$, 1.16 mM $MgCl_2$, 1.2 mM KH_2PO_4 , 12.4 mM $NaHCO_3$ and 5.0 mM glucose and gassed with 95% O_2 –5% CO_2 at 30°C. The right atrium was separated from the ventricle, left atrium and other tissues. The atrial preparations were transferred to an organ bath containing 2.5 ml Krebs–Henseleit solution and were connected to an isometric transducer (model UL-2GR; Minebea, Nagano) by a silk thread under a resting tension of 1 mN.

Experimental Procedures The atrial preparations were equilibrated for 1 h to stabilize the rate of contraction. Concentration–response curves for aconitine (0.10–0.22 μ M) were obtained by cumulatively applying aconitine every 2 min to the organ bath. The response of the atria to each concentration of aconitine became constant within this time interval. The rate of contraction was monitored on a recorder (Recti-Horiz-8K, San-ei) and calculated by measuring the time required for 20 beats within the last 10 s before the next addition of aconitine. Higenamine, practolol, butoxamine, forskolin, or a combination of higenamine with practolol or forskolin were added to the organ bath 5 min before the cumulative addition of aconitine and remained in the organ bath throughout the experiments. Similarly, cumulative concentration–response curves for higenamine were obtained in the presence of aconitine and the rate of contraction was measured. A single concentration–response curve was obtained from one atria.

For cholera toxin experiments, isolated atria were equilibrated in Krebs–Henseleit solution containing 50 and 100 nM CTX for 1 h and then were completely washed out. An increase in either the concentration of CTX to

more than 100 nM or in the treatment time to more than 1 h caused irregular beating of the right atria. Both the concentrations and treatment times for CTX are very similar to those required to increase the cyclic AMP level 2.4 times in chick embryo right ventricles¹⁴⁾ and affect the isoproterenol-induced positive inotropic effect in guinea pig atria.¹⁵⁾ Forskolin (15–100 nM) was used to activate adenylyl cyclase. At similar concentrations it causes a significant increase in adenylyl cyclase activity in rat heart¹⁶⁾ and potentiates isoproterenol-induced positive inotropic effects in guinea pig atria.¹⁵⁾

PTX (75–150 µg/kg, as a bolus) was injected intraperitoneally (i.p.) into the mice 3 d before the isolation of the atria. PTX at these doses did not produce apparently any toxic effects on mice.

Drugs The following drugs were used: aconitine (Sigma Chemical, St. Louis, MO, U.S.A.); higenamine HCl (provided by Prof. Momose, Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University); practolol, butoxamine and forskolin (Kaken, Tokyo); CTX (Calbiochem-Behring, CA, U.S.A.) and PTX (Kaken, Tokyo). Aconitine was first dissolved in 0.1 N HCl (100 µl) and then diluted with distilled water to give a 1 mM stock solution. Forskolin was dissolved in 99.5% ethanol and then diluted with distilled water. The final concentration of ethanol in the organ bath was 0.02%, which did not affect the rate of contraction of the right atria.

Statistics Data were expressed as mean ± S.E.M. Significant differences were evaluated by means of one-way analysis of variance (ANOVA) and the multiple comparison test of Scheffe or Tukey. Differences were considered to be significant when the *p* value was smaller than 0.05 or 0.01.

RESULTS

Positive Chronotropic Effects of Aconitine and Higenamine, and Their Mutual Potentiating Effects Aconitine (0.10–0.22 µM) induced a positive chronotropic effect that

was followed by arrhythmia and bigeminy (at 0.16–0.22 µM) in isolated right atria. The positive chronotropic effect reached a peak at 0.16 µM aconitine and then decreased with subsequent increases in concentration (Fig. 1A). Higenamine alone at 5 nM having no effect on the basal frequency (Table 1), significantly potentiated the positive chronotropic effect of aconitine and shifted the concentration–response curve to the left (Fig. 1A) as already reported by us,¹⁰⁾ and did not induce any bigeminy or arrhythmia. Aconitine (25 nM) did not affect the basal frequency (control: 4.57 ± 0.05 Hz, *n* = 5; aconitine 10 nM: 4.7 ± 0.08 Hz, *n* = 4; and 25 nM: 4.67 ± 0.08 Hz, *n* = 4) of the isolated right atria, but significantly potentiated the positive chronotropic effect of higenamine (Fig. 1B). Aconitine (10 nM) tended to potentiate the higenamine effect. The maximum response of higenamine in the concentration–response curve was not changed by aconitine.

Blockade by Practolol of the Potentiation by Higenamine of the Aconitine-Induced Positive Chronotropic Effect The influence of practolol, a selective β_1 -adrenergic antagonist, on the potentiation by higenamine of the aconitine-induced positive chronotropic effect was investigated. Practolol at 1 nM blocked the potentiation by higenamine of the aconitine action, but it did not affect the effect of aconitine alone (Fig. 2A). However, practolol at 30 and 300 nM shifted the concentration–response curve of aconitine to the right (Fig. 2B) without affecting the basal frequency (control: 5.05 ± 0.09 Hz, *n* = 9; practolol 30 nM: 5.11 ± 0.19 Hz, *n* = 5; and 300 nM: 5.14 ± 0.13 Hz, *n* = 5). Three atria out of five recovered from aconitine-induced bigeminy at 300 nM but not at 30 nM practolol. In contrast, butoxamine (1 µM), a selective β_2 -antagonist did not affect the positive chronotropic effect of aconitine and the appearance of bigeminy (data not shown).

Depression of Aconitine-Induced Positive Chronotropic Effect by Higenamine in CTX- and Forskolin-Treated Atria and Its Abolition by PTX The effects of CTX and forskolin were examined on the positive chronotropic interaction between aconitine and higenamine in isolated right atria of mice. Both CTX (50 and 100 nM) and

Fig. 1. Potentiation by Higenamine of the Aconitine-Induced Positive Chronotropic Effect (A), and by Aconitine of the Higenamine-Induced Positive Chronotropic Effect (B) in Isolated Right Atria of Mice

Higenamine (5 nM, closed circles in A) was applied to the organ bath 5 min before the cumulative application of aconitine, and, similarly, aconitine (10 and 25 nM, closed circles in B) was applied before the cumulative application of higenamine. Both preadministered higenamine and aconitine were present throughout the experiments. All the values are means ± S.E.M (*n* = 5–9). ***p* < 0.01: significantly different from the corresponding control values (open circles) without higenamine (in A) or aconitine (in B) by means of ANOVA and the multiple comparison test of Tukey or Scheffe.

Fig. 2. Practolol-Induced Blockade of the Potentiating Positive Chronotropic Interaction between Aconitine and Higenamine (A), and Its Competitive Antagonism of the Effects of Aconitine Alone (B) in Isolated Right Atria of Mice

In A, the concentration–response curves for aconitine action were obtained in the presence of practolol (1 nM) (open circles) and a combination of practolol (1 nM) and higenamine (5 nM) (closed circles). In B, concentration–response curves for aconitine were obtained in the absence (open circles) and presence (closed circles) of practolol (30 and 300 nM). All the values are means $\pm$ S.E.M ($n=5-9$). * $p<0.05$ or ** $p<0.01$: significantly different from the corresponding values without practolol by means of ANOVA and the multiple comparison test of Scheffe.

Table 1. No Changes in the Frequency Produced by Higenamine in the Isolated Right Atria of Mice Following Treatment with CTX, Forskolin and PTX

Treatment	Frequency (Hz)
Normal atria	
Control	5.05 $\pm$ 0.11
Higenamine 5 nM	5.10 $\pm$ 0.20
CTX 50 nM	5.60 $\pm$ 0.20
100 nM	5.89 $\pm$ 0.32
CTX 100 nM + higenamine 5 nM	6.42 $\pm$ 0.14
Forskolin 15 nM	4.93 $\pm$ 0.20
30 nM	5.19 $\pm$ 0.07
100 nM	5.34 $\pm$ 0.16
Forskolin 100 nM + higenamine 5 nM	5.56 $\pm$ 0.13
PTX 150 μ g/kg (i.p., 3 d)	
Control	4.80 $\pm$ 0.20
CTX 100 nM	5.51 $\pm$ 0.14
CTX 100 nM + higenamine 5 nM	5.70 $\pm$ 0.16
Forskolin 100 nM	5.48 $\pm$ 0.22
Forskolin 100 nM + higenamine 5 nM	5.75 $\pm$ 0.15

All values are mean $\pm$ S.E.M. ($n=4-9$).

forskolin (15–100 nM) tended to increase the basal frequency of the isolated right atria of mice (Table 1). Furthermore, CTX (100 nM, 1 h) and forskolin (100 nM) did not affect significantly the effect of higenamine (5 nM) on the basal frequency (Table 1).

The potentiating interaction between aconitine and higenamine was changed to a depressive interaction by pretreating with CTX and forskolin. Higenamine at 5 nM did not potentiate the aconitine action in atria pretreated with 50 nM CTX (data not shown) and significantly depressed it with 100 nM CTX (Fig. 3A, upper panel). A similar pattern in the interaction between aconitine and higenamine was observed in the presence of forskolin. The positive chronotropic effect of aconitine was significantly depressed by higenamine (5 nM) in the presence of 100 nM and 30 nM forskolin but not 15 nM forskolin (Fig. 3A, lower panel and data not shown). In the absence of higenamine, neither CTX (50 and 100 nM) nor forskolin (30 and 100 nM) significantly affected the positive chronotropic effect of aconitine (Fig. 4). The occurrence of bigeminy was not

changed by CTX and forskolin.

The effects of CTX and forskolin on the positive chronotropic interaction between aconitine and higenamine were further examined in the isolated right atria of PTX-pretreated (150 μ g/kg, i.p., 3 d) mice. In these atria, the basal frequency was slightly reduced compared with that in normal atria. Furthermore, similar to the normal atria, CTX (100 nM, 1 h) and forskolin (100 nM) tended to increase the basal frequency which did not significantly affect the effect of higenamine (5 nM) (Table 1). However, PTX-pretreatment abolished the CTX (100 nM, 1 h)- and forskolin (100 nM)-effects on the aconitine–higenamine interaction. No depression of the aconitine-induced positive chronotropic effect by higenamine was observed in the presence of CTX (Fig. 3B, upper panel) and forskolin (Fig. 3B, lower panel). Furthermore, the potentiation of the aconitine-induced positive chronotropic effect by higenamine (5 nM) tended to recover despite the presence of CTX (Fig. 3B, upper panel). Typical data showing the depressive action of higenamine (5 nM) on 0.14 μ M aconitine in CTX- and forskolin-treated atria and its abolition by PTX-pretreatment are shown in Fig. 5. However, in PTX (150 μ g/kg)-pretreated atria (without any intervention by CTX or forskolin) higenamine potentiated the aconitine action just as in normal atria. The potentiation occurred at higher concentrations (Fig. 6A). PTX at 150 μ g/kg (i.p., 3 d) significantly depressed the aconitine-induced positive chronotropic effect, while at 75 μ g/kg it tended to depress the aconitine action (Fig. 6B). No change in aconitine-induced bigeminy was observed following PTX-pretreatment.

DISCUSSION

We investigated the mechanisms of the potentiative chronotropic interaction between aconitine and higenamine in isolated right atria of mice by using CTX, forskolin and PTX.

Aconitine and higenamine have different sites of action in the myocardial cells. Aconitine acts on the receptor site II of sodium channels like batrachotoxin, veratridine, and

Fig. 3. Inhibitory Effects of Higenamine on the Aconitine-Induced Positive Chronotropic Effect in CTX (A, Upper Panel)- and Forskolin (A, Lower Panel)-Treated Isolated Right Atria of Mice and Their Antagonism by PTX (B, CTX: Upper Panel; Forskolin: Lower Panel)

The atrial preparations were pretreated with CTX (100 nM, 1 h) and then were washed out. Forskolin (100 nM) and higenamine (5 nM, closed circles) were applied 5 min before the cumulative application of aconitine and were present throughout the experiments. PTX (150 µg/kg, i.p.) was injected into the mice 3 d before the isolation of the atria. All values are means $\pm$ S.E.M ($n=4-7$). * $p<0.05$ or ** $p<0.01$: significantly different from the corresponding control value without higenamine (open circles) by means of ANOVA and the multiple comparison test of Scheffe.

Fig. 4. CTX (A)- and Forskolin (B)-Pretreatment Does Not Affect the Positive Chronotropic Effect of Aconitine in Isolated Right Atria of Mice

In A, the atrial preparations were pretreated with CTX (50 nM, triangles; and 100 nM, closed circles) and then washed out. In B, forskolin (30 nM, triangles; and 100 nM, closed circles) was applied 5 min before the cumulative application of aconitine and was present throughout the experiments. All values are means $\pm$ S.E.M ($n=4-9$). No significant differences from the corresponding control value (open circles) were observed by means of ANOVA and the multiple comparison test of Scheffe.

grayanotoxin I in neural and cardiac cells.⁹⁾ It causes a delay in the final repolarization phase of the cardiac action potential which initiates premature excitations leading to tachyarrhythmias¹⁷⁾ as a result of prolongation of the open state of sodium channels.⁹⁾ *In vivo* aconitine-induced tachyarrhythmia is mediated through a central β -adrenergic mechanism.⁶⁾ However, the involvement of a peripheral β -adrenergic mechanism in aconitine-induced tachyarrhythmia is still unclear. Higenamine causes positive chronotropic and inotropic effects through activation of β_1 -adrenergic receptors¹⁰⁾ and increases cyclic AMP in isolated rat atria.¹⁸⁾

Higenamine potentiates the aconitine-induced positive

chronotropic effect in isolated right atria of mice as we have reported previously.¹⁰⁾ Conversely, aconitine alone having no effect on the basal frequency of the right atria, potentiated the positive chronotropic effect of higenamine. Furthermore, the blocking action of practolol (1 nM) on the higenamine-induced potentiation of aconitine action, suggests that the aconitine-higenamine interaction involves the participation of β_1 -adrenergic receptors. Moreover, the aconitine-induced positive chronotropic effect was competitively antagonized by practolol (30 and 100 nM), but not by butoxamine, a β_2 -adrenergic antagonist. Aconitine may act directly on the myocardial β_1 -adrenergic receptors rather than through the presynap-

tic release of catecholamines,⁵⁾ because reserpine pretreatment does not interfere with the positive chronotropic and inotropic effects of aconitine in cat and guinea pig papillary muscles.^{19,20)}

CTX activates G_s -protein through an irreversible ADP-ribosylation of the protein.^{21,22)} G_s -protein is coupled with adenylyl cyclase and is responsible for the β -adrenergic receptor-stimulated increase in cyclic AMP that is associated with an increased rate and force of cardiac contraction.²³⁾ Forskolin stimulates cyclic AMP formation and potentiates agonist-stimulated cyclic AMP formation.²⁴⁾ The potentiation effect of forskolin is in part

modulated by activation of G_s -protein. CTX and forskolin, owing to their stimulating effects on the β_1 -adrenergic- G_s -adenylyl cyclase system, were assumed to augment the higenamine-induced potentiation of aconitine action but, interestingly, they changed the pattern of aconitine-higenamine interaction ultimately leading to the depression of aconitine action by higenamine. Both CTX and forskolin did not induce a significant positive chronotropic effect by themselves and also did not affect the positive chronotropic effect of aconitine alone (in the absence of higenamine). In this regard we consider that much less activation of G_s -protein and adenylyl cyclase is sufficient to affect the higenamine-induced potentiation of aconitine action. From these results we suggest that the positive chronotropic interaction between aconitine and higenamine may be modulated by the β_1 -adrenergic receptor- G_s -adenylyl cyclase system.

Some other mechanism may contribute to the suppression of aconitine action by higenamine in CTX- and forskolin-treated atria. We assume that G_i -protein may be involved in the mechanism. G_i -protein interacts functionally with the components of the β -adrenergic signalling pathway.^{23,25)} Moreover, we have shown that CTX accentuates the muscarinic inhibition of the positive chronotropic effect of higenamine through an interaction with G_i -protein.²⁶⁾ PTX functionally inactivates the muscarinic receptors through the inhibition of G_i -protein.²⁷⁾ We observed that PTX-pretreatment abolished the depression of the aconitine-induced positive chronotropic effect by higenamine in CTX- and forskolin-treated atria. Therefore, following pre-application of CTX or forskolin, the higenamine-aconitine interaction may lead to the activation (or functional changes) of G_i -protein which in turn interacts with adenylyl cyclase or β_1 -adrenergic receptors causing a depression of aconitine action. On the basis of this postsynaptic interaction between the β_1 -adrenergic signalling pathway and G_i -protein, the aconitine-higenamine interaction may be explained by accentuated antagonism. Previously, we have reported that concomitant activation of both β_1 -adrenergic receptors and the receptor-coupled G_s -protein

Fig. 5. Typical Recordings Showing the CTX- and Forskolin-Induced Inhibitory Effect on the Potentiative Chronotropic Interaction between Higenamine and Aconitine ($0.14 \mu\text{M}$) (Panel A) and Its Antagonism by PTX ($150 \mu\text{g/kg}$, i.p., 3 d) (Panel B) in Isolated Right Atria of Mice

Fig. 6. PTX-Pretreatment Does Not Affect the Potentiating Interaction between Aconitine and Higenamine (A), but Depresses the Aconitine-Induced Positive Chronotropic Effect in Isolated Right Atria of Mice

PTX (75 – $150 \mu\text{g/kg}$, i.p.) was injected into the mice 3 d before isolation of the atria. In A, concentration-response curves for aconitine were obtained in atria pretreated with $150 \mu\text{g/kg}$ PTX in the presence (closed circles) or absence (open circles) of higenamine (5 nM). Higenamine was applied 5 min before the cumulative application of aconitine and was present throughout the experiments. In B, concentration-response curves for aconitine were obtained in the atria pretreated with 75 and $150 \mu\text{g/kg}$ PTX (closed circles) and compared with that in normal atria (open circles). All values are means $\pm$ S.E.M ($n=5$). * $p<0.05$ or ** $p<0.01$: significantly different from the corresponding control value by ANOVA and the multiple comparison test of Tukey.

may cause accentuation of G_i -protein-mediated effects.²⁶⁾ The upregulation of G_i -protein occurs in a variety of cell types in response to stimulation of G_s -protein coupled receptors or in response to stimulation with forskolin.^{28,29)} Thus, during the aconitine-higenamine interaction, CTX and forskolin may stimulate G_i -protein through a common cyclic AMP-dependent mechanism. An increase in intracellular cyclic AMP promotes $G_i\alpha$ -2-gene transcription leading to $G_i\alpha$ -protein-upregulation.³⁰⁾ Conversely, G_i -protein is associated with an inhibition of adenylyl cyclase.³¹⁾ Moreover, G_i -protein interacts with G_s -protein and reduces β -receptor affinity for its agonist in S49 lymphoma cell membranes.²⁵⁾ In reconstitution experiments, addition of pure G_i -protein to a mixture of pure β -adrenergic receptors and catalyst attenuates the β -adrenergic receptor-dependent activity.³²⁾ PTX-catalysed ADP-ribosylation of G_i -proteins inhibits the ability of G_i to associate with the β -adrenergic receptor- G_s -adenylyl cyclase complex and nullifies its effect on β -adrenergic receptors. These findings also explain our observation that in normal atria (CTX- and forskolin-untreated) PTX-pretreatment did not change the aconitine-higenamine potentiating interaction. We suggest that when G_s -protein and adenylyl cyclase remain unstimulated, G_i -protein inhibition by PTX does not necessarily affect the β_1 -adrenergic receptor-mediated potentiation of aconitine action by higenamine. The action of aconitine alone (without higenamine, CTX or forskolin) is inhibited in the atria from PTX-treated mice which may be due to the uncoupling of muscarinic receptors from G_i -protein. Muscarinic antagonists also antagonize the tachyarrhythmic effect of aconitine.¹⁰⁾ Aconitine causes the release of ACh from the presynaptic neurones³³⁾ which may contribute to the induction of tachyarrhythmia. Cholinergic agonists and vagal stimulation potentiate the inducibility of tachyarrhythmia in the isolated canine right atrium through the shortening of the effective refractory period.⁸⁾ Therefore, the positive chronotropic effect of aconitine which is accompanied by arrhythmia is, at least in part, mediated through the muscarinic- G_i -protein system, although the precise mechanism for this is unclear.

In conclusion, the potentiating interaction between aconitine and higenamine with respect to the positive chronotropic effect in isolated right atria of mice involves a cross-talk between the β_1 -adrenergic signalling pathway and G_i -protein.

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