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K^+_{ACh} channel activation with carbachol increases atrial ANP release

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ABSTRACT

Although it has been known that atrial natriuretic peptide (ANP) release is regulated through muscarinic acetylcholine receptors (mAChR), the mechanism by which this neurotransmitter regulates atrial ANP release is largely unknown. This study tested the hypothesis that K^+_{ACh} channels mediate the action of mAChR on atrial myocyte ANP release. Experiments were performed in perfused beating rabbit atria. Carbachol (CCh), an agonist of cardiac mAChR, increased atrial myocyte ANP release concomitantly with a decrease in stroke volume and intra-atrial pulse pressure in a concentration-dependent manner. Isoproterenol, a β-adrenoceptor agonist, decreased ANP release concomitantly with an increase in cAMP and mechanical dynamics. In the presence of isoproterenol, the CCh-induced increase in ANP release and decrease in cAMP efflux levels and mechanical dynamics were able to be repeated. The CCh-induced changes were blocked by selective M_2 mAChR antagonists. Tertiapin, a selective G-protein-gated K^+_{ACh} channel blocker, attenuated the CCh-induced increase in ANP release and decrease in mechanical dynamics in a concentration-dependent manner, but without a significant effect on the CCh-induced decrease in cAMP efflux levels. The CCh-induced changes in ANP release and atrial dynamics were inhibited in the atria from pertussis toxin-pretreated rabbits. These findings demonstrate that G-protein-gated K^+_{ACh} channels regulate atrial myocyte ANP release. The present study also shows that mAChR and adrenoceptors have opposing roles in the regulation of ANP release.
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Introduction

Since the discovery by De Bold and his colleagues of atrial natriuretic peptide (ANP), the heart has been considered as an endocrine gland (De Bold, 1985). However, no specific factor for the regulation of ANP release has yet been identified. Both adrenoceptors and muscarinic acetylcholine receptors (mAChR) have been shown to be involved in the regulation of ANP release. Some reports have demonstrated that stimulation of either adrenoceptors (Ruskoaho et al., 1985; Schiebinger et al., 1987; Sonnenberg and Veress, 1984) or mAChR (Inoue et al., 1988; Ruskoaho et al., 1985; Sonnenberg and Veress, 1984) increases ANP release, while others have shown that stimulation of either adrenoceptors (Inoue et al., 1988) or mAChR (Schiebinger et al., 1987) decreases release in perfused heart and isolated atria. Thus, the reports on the roles of adrenoceptors and mAChR for the control of ANP release have been contradictory. The exact nature of the role of, and interrelation between, adrenoceptors and mAChR for the regulation of ANP release remain to be clarified. Furthermore, the mechanism by which stimulation of mAChR regulates ANP release is not well defined. In the present study, we attempted to address these questions by using a method allowing the measurement of changes in the volume of extracellular fluid (ECF) translocated across the endocardial atrial wall, along with stroke volume and pulse pressure in the beating rabbit atria. Using this method, it is possible to dissociate the rate of myocyte ANP release from the atrial ANP secretion into the atrial lumen (which is dependent on mechanical dynamic changes of the atrium), which may explain, in part, the contradictory results previously reported.

Because the mechanism by which mAChR activation regulates atrial ANP release is not well defined and the K^+_{ACh} channel activation is an important pathway in the signaling of cardiac muscarinic receptors (Brodde and Michel, 1999; Yamada et al., 1998), we hypothesized that G-protein-gated K^+ channels are involved in the muscarinic regulation of atrial ANP release. Therefore, the purpose of the present study was to test the effects of agonists of mAChR on ANP release and its modulation by M_2 mAChR antagonists, a G-protein-gated K^+_{ACh} channel inhibitor (Jin and Lu, 1998), and a Gαi/o inhibitor. Experiments were performed in perfused beating rabbit atria, allowing measurements of changes in ANP release, cAMP efflux and mechanical

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dynamics. The present study showed for the first time that G-protein-gated K^{+}_{ACh} channels are involved in the regulation of atrial myocyte ANP release.

Materials and methods

The investigation conformed to the “Principles of laboratory animal care” (NIH publication No. 86-23, revised 1985).

Beating perfused atrial preparation

An isolated atrial preparation was set up using the method described previously (Cho et al., 1995). To maintain the homeostatic balance between the variable ion channels and the excitability of the atrial myocytes, the preparation was electrically paced (1.3 Hz). Briefly, the hearts were rapidly removed and placed in oxygenated warm saline. The left atrium was then dissected. A calibrated transparent atrial cannula, containing two small catheters within it, was inserted into the left atrium through the atrioventricular orifice. The outer tip of the atrial cannula was open to allow for outflow from the atrium. One of the two catheters located in the atrium was for inflow, and the other catheter was used to record pressure changes in the atrium. The cannulated atrium was then transferred to an organ chamber containing buffer at 36.5 °C. The pericardial space of the organ chamber was open to the air so as not to restrict atrial dynamics. The atrium was immediately perfused with HEPES-buffered solution by means of a peristaltic pump (1 ml/min). The changes in atrial stroke volume were monitored by reading the lowest level of the water column in the calibrated atrial cannula during end diastole. Atrial pulse pressure was measured via a pressure transducer connected to the intra-atrial catheter and recorded on a physiograph.

Measurement of the translocation of extracellular fluid (ECF)

To calculate the real rate of myocyte ANP release into the extracellular space surrounding the atrial myocytes (*vide infra*), we estimated the amount of transendocardial ECF translocation. This was done by measuring the transmural atrial clearance of [^{3}H]inulin, as described previously (Cho et al., 1995). Radioactivity in the atrial perfusate and pericardial buffer solution was measured using a liquid scintillation system, and the amount of ECF translocated through the atrial wall was calculated as follows:

ECF translocated (μl min^{-1} g atrial wet wt^{-1})=(total radioactivity in the perfusate (cpm min^{-1})×1000/(radioactivity in the pericardial reservoir (cpm μl^{-1})×atrial wet wt (mg).

Experimental protocols

The beating atria were perfused for 60 min to stabilize ANP secretion. [^{3}H]inulin was introduced to the pericardial fluid 20 min before the start of the sample collection (Cho et al., 1995; Wen et al., 2004). The perfusate was collected for analyses at 2-min intervals at 4 °C. Experiments were carried out using 22 groups of atria (Fig. 1), to answer the following questions.

Do mAChR agonists increase ANP release?

An initial control period (48 min, comprising four 12-min period) was followed by an infusion of carbachol (CCh), an agonist of mAChR (0.1 μM, group *1*, *n*=3; 0.3 μM, group *2*, *n*=7; 1.0 μM, group *3*, *n*=9; 3.0 μM, group *4*, *n*=9) for 36 min (Fig. 2). To evaluate the effects of CCh, the values (mean of two fractions) obtained before and after the addition at 12, 24 and 36 min were compared. The data were expressed as percent differences from the control (before the addition of CCh).

In another series of experiments, the control period (36 min) was followed by an infusion of CCh. CCh (1.0 μM) was administered for 12 min, three times, with intervals of 24 min between each. At the third exposure to CCh, pretreatment with the vehicle for 24 min was followed by the addition of CCh in the continuous presence of vehicle (vehicle, group *5*, *n*=8, Fig. 3A).

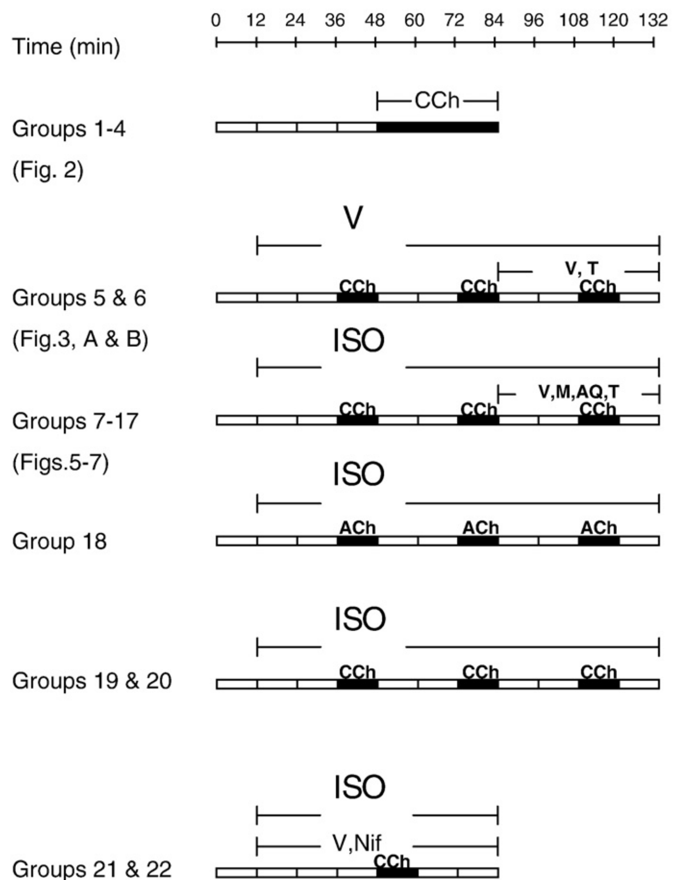

Fig. 1. Protocols for present experiments. Atria were paced at 1.3 Hz. The values (mean of two fractions) obtained before and after the addition of carbachol (CCh) or acetylcholine (ACh) were compared. V, vehicle; T, tertiapin; ISO, isoproterenol; M, methoctramine; AQ, AQ-RA 741; Nif, nifedipine. See Experimental protocols for details.

Does adrenoceptor stimulation decrease ANP release and affect CCh-induced ANP release?

In the heart, adrenergic effects are mainly exerted through the ß-adrenoceptors. To compare the effects of muscarinic stimulation with the adrenergic influence on the ANP release, administration of a ß-adrenoceptor agonist, isoproterenol (ISO, 2 nM), for 24 min was followed by three exposures to CCh in the continuous presence of ISO (ISO plus vehicle, group *7*, *n*=9, Fig. 5A). In another series of experiments, 24 min of ISO was followed by administration of acetylcholine (ACh, 3.0 μM) in the continuous presence of ISO (ISO plus ACh, group *18*, *n*=3).

Are the effects of muscarinic stimulation with CCh on ANP release mediated by the M_2 mAChR?

At the third exposure to CCh, pretreatment with either of two selective M_2 mAChR antagonists for 24 min was followed by administration of CCh in the presence of the corresponding antagonist [methoctramine or AQ-RA 741 (ISO plus methoctramine: methoctramine 0.1 μM, group *8*, *n*=2, Fig. 6; methoctramine 0.3 μM, group *9*, *n*=3, Fig. 6; methoctramine 1.0 μM, group *10*, *n*=3, Fig. 6; ISO plus AQ-RA 741: AQ-RA 741 0.1 μM, group *11*, *n*=5, Fig. 6)]. The concentrations of methoctramine and AQ-RA 741 used were in the range described in a previous study (Dorje et al., 1991), and had been shown to have selectivity for the M_2 mAChR.

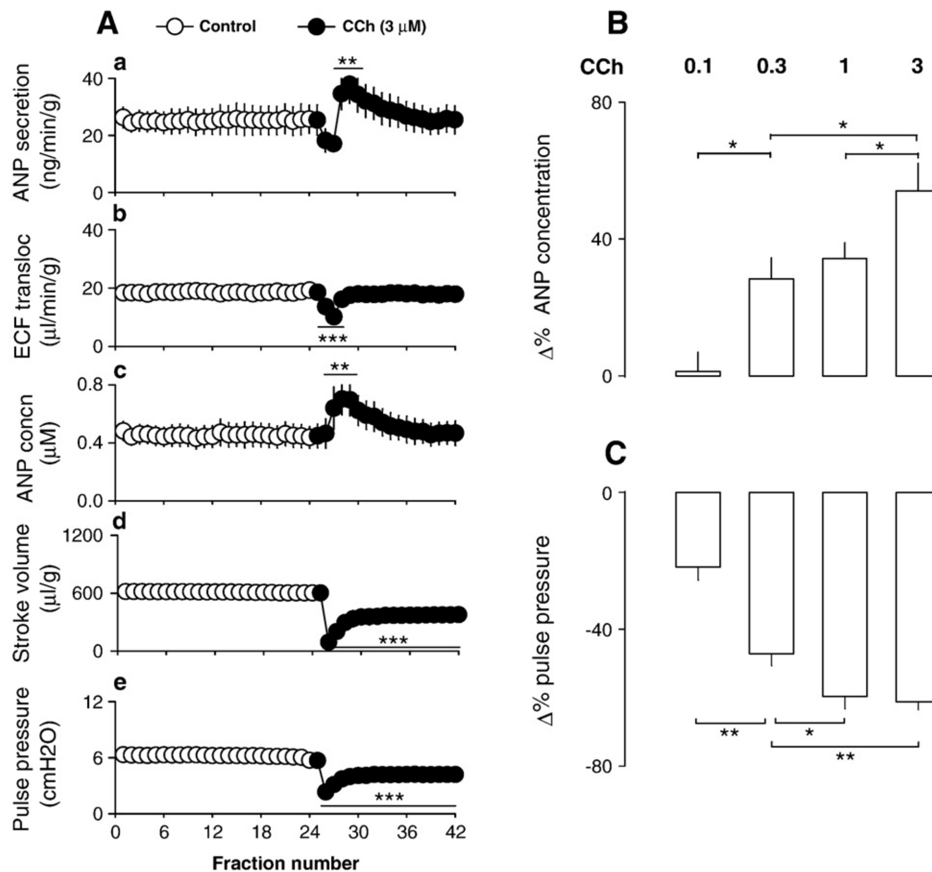

Fig. 2. Effects of carbachol on the levels of ANP secretion, extracellular fluid translocation and atrial dynamics in beating rabbit atria. (A) effects of carbachol (CCh) on ANP secretion (a), extracellular fluid (ECF) translocation (b), ANP concentration (c), stroke volume (d), and pulse pressure (e) (n=9). (B) concentration-dependent increase in ANP concentration by CCh. CCh, 0.1 μM (n=3), 0.3 μM (n=7), 1.0 μM (n=9), 3.0 μM (n=9). Data were expressed as difference in percent changes over the control. (C) concentration-dependent decrease in pulse pressure by CCh. CCh, 0.1 μM (n=3), 0.3 μM (n=7), 1.0 μM (n=9), 3.0 μM (n=9). *p<0.05, **p<0.01, ***p<0.001 vs. the values before the addition of CCh or corresponding values.

Are the effects of muscarinic stimulation with CCh on ANP release mediated by K^+_{ACh} channels?

At the third exposure to CCh, pretreatment with a selective K^+_{ACh} channel blocker, tertiapin, for 24 min was followed by administration of CCh in the presence of this blocker (in the absence of ISO, vehicle plus tertiapin: tertiapin, 0.1 μM, group *6*, n=7, Fig. 3B; in the presence of ISO, ISO plus tertiapin: tertiapin 0.003 μM, group *12*, n=2, Fig. 7A; tertiapin 0.01 μM, group *13*, n=7, Fig. 7A; tertiapin 0.03 μM, group *14*, n=8, Fig. 7A; tertiapin 0.1 μM, group *15*, n=8, Figs. 5B and 7A; tertiapin 0.3 μM, group *16*, n=8, Fig. 7A; teriapin 0.6 μM, group *17*, n=7, Fig. 7A). Tertiapin is a potent and selective G-protein-gated K^+_{ACh} channel blocker (Jin and Lu, 1998). In the present study, we used tertiapinQ, in which methionine residue 13 in tertiapin is replaced by glutamine (Jin and Lu, 1999). TertiapinQ is known to be stable and functionally similar to native tertiapin.

Are the effects of CCh related to the pertussis toxin-sensitive G-proteins?

For the treatment of pertussis toxin (PTX), rabbits were intravenously injected with PTX (5 μg/kg) or vehicle 72 h before isolation of atria. The dose of PTX and duration of the treatment were in the range previously reported (Endoh et al., 1985). The dose and duration of PTX treatment effectively blocked the CCh-induced activation of cardiac mAChR. Atria from rabbits pretreated with PTX or vehicle were infused with ISO, followed by CCh (PTX-pretreated, group *19*, n=9; sham-treated, group *20*, n=3).

To evaluate the effects of vehicle or modulators on the CCh-induced changes in atrial ANP release and pulse pressure, the values obtained at the second and third exposures to CCh were compared. The second exposure to CCh in the presence of vehicle was used as control, and the third exposure to CCh in the presence of vehicle or modulator was used as control or experimental value, respectively. The data, expressed as percent differences over the control, were compared between groups treated with vehicle and with modulator. Percent differences=$[(a3-b3)-(a2-b2)]\times 100/(a2-b2)$, where $a3$ and $b3$, and $a2$ and $b2$ were the actual values obtained after (a) and before (b) the addition of CCh at the third and second exposures, respectively.

Are the effects of CCh related to L-type Ca^{2+} channel activity?

To define the role of Ca^{2+} entry via L-type Ca^{2+} channels, nifedipine (1 μM) was applied. Thirty-six min of nifedipine or vehicle (in the presence of ISO) was followed by 12 min of CCh in the continuous presence of ISO. Values obtained before and after CCh were compared (ISO plus nifedipine, group *21*, n=6; ISO plus vehicle, group *22*, n=5). The data were expressed as percent differences over the control (before the addition of CCh).

Radioimmunoassay of ANP

Immunoreactive ANP in the perfusate was measured by a specific radioimmunoassay, as described previously (Cho et al., 1995). The amount of ANP secreted was expressed as nanograms of ANP per minute per gram of atrial tissue. The molar concentration of immunoreactive ANP in terms of ECF translocation reflects the concentration of ANP in the interstitial space of the atrium and, therefore, indicates the rate of myocyte release of ANP into the

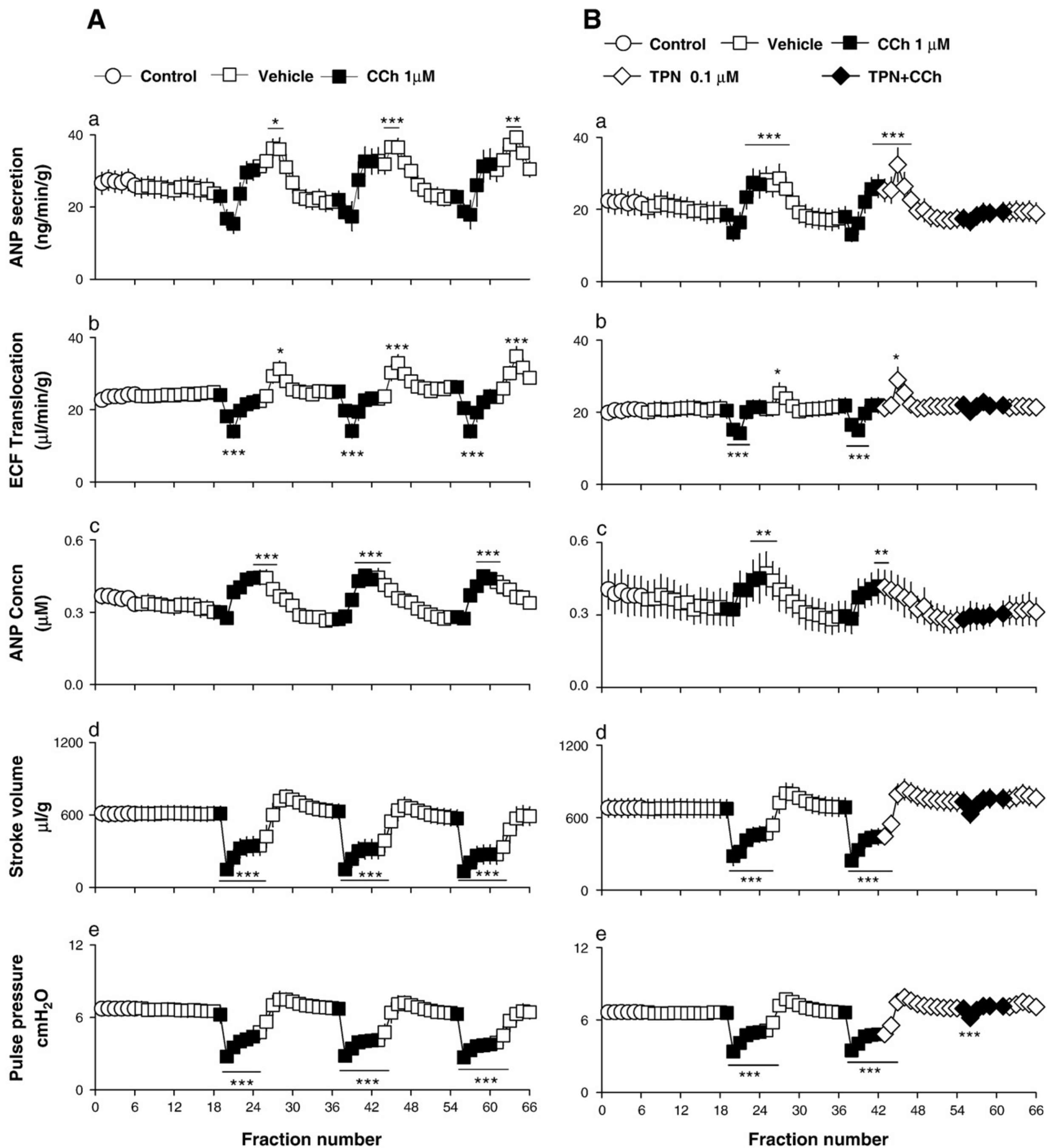

Fig. 3. Effects of carbachol and its modulation by tertiapin on ANP release and atrial dynamics in beating rabbit atria. (A) effects of carbachol (CCh) on ANP secretion (a), ECF translocation (b), ANP concentration (c), stroke volume (d), and pulse pressure (e) ($n=8$). (B) modulation by tertiapin (TPN) of the CCh-induced changes in ANP secretion (a), ECF translocation (b), ANP concentration (c), stroke volume (d), and pulse pressure (e) ($n=7$). $^{**}p<0.01$, $^{***}p<0.001$ vs. the values before the addition of CCh.

surrounding paracellular space (Cho et al., 1995). It was calculated by the following formula: ANP released (μM)=immunoreactive ANP (in pg min^{-1} g^{-1})/ECF translocated (in μl min^{-1} g^{-1})·3063 [mol wt of ANP-(1–28)].

Radioimmunoassay of cAMP

cAMP was measured by equilibrated radioimmunoassay (Cui et al., 2002; Wen et al., 2004). The level of cAMP efflux was expressed as pmol of cAMP per min g of atrial tissue. The level of cAMP efflux has been shown to be linearly correlated with the atrial tissue content of cAMP (Wen et al., 2004). The molar concentration of cAMP efflux in terms of ECF translocation, which may reflect the concentration of cAMP in the interstitial space fluid (Cui et al., 2002; Wen et al., 2004), was calculated as cAMP efflux concentration (μM); cAMP (in pmol min^{-1} g^{-1})/ECF translocated (in μl·min^{-1} g^{-1}). Nonspecific binding was <2.0%. The intra- and interassay coefficients of variation were 5.0 ($n=10$) and 9.6% ($n=10$), respectively.

Statistical analysis

Significant difference was compared using repeated measures ANOVA followed by Bonferroni's multiple-comparison test (see Figs. 2–6). Student's *t*-test for unpaired or paired data (see Figs. 6 and 7B)

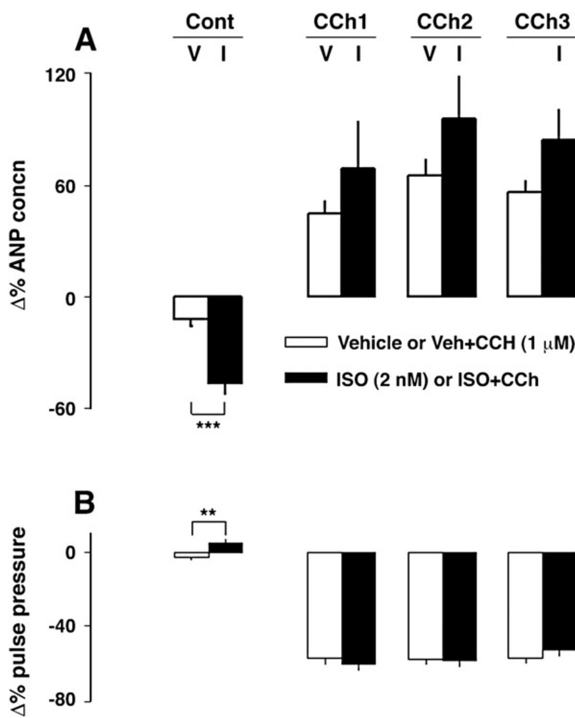

Fig. 4. Comparisons of effects of repeating carbachol administration in the presence and absence of isoproterenol. (A) differences in percent changes over the control in ANP concentration. (B) differences in percent changes over the control in pulse pressure. Data were derived from Figs. 3 and 5. Data were expressed as differences in percent changes over the control (before the addition of carbachol, CCh). Cont, control period; CCh1, CCh2, and CCh3, first, second, and third exposures of CCh, respectively. V, vehicle; I, isoproterenol. $^{**}p<0.01$, $^{***}p<0.001$.

was also applied. Statistical significance was defined as $p<0.05$. The results are given as means ± SE.

Results

Do mAChR agonists increase ANP release?

Atrial ANP secretion, the concentration of ANP in the perfusate, in terms of the ECF translocation, which reflects the rate of atrial myocyte ANP release, and atrial dynamics (atrial stroke volume and pulse pressure) were steady and stable during the control periods (Fig. 2A). CCh increased ANP secretion and ANP concentration (ANP release) concomitantly with decreases in ECF translocation and atrial dynamics. The CCh-induced increase in ANP release was rapid, but was not maintained (showing a decline after 12 min), despite the continuous presence of CCh, slowly returning toward control levels (Fig. 2Aa and Ac). The CCh-induced decrease in atrial dynamics was significant during the presence of CCh (Fig. 2Ad and Ae). All CCh-induced responses were concentration-dependent (Fig. 2B and C). The CCh-induced increase in ANP release and decrease in atrial dynamics were dissociated during the later stages of treatment and at lower concentrations of CCh (Fig. 2).

We then tested the effect of repeating CCh administration. CCh was administered for 12 min, three times, at intervals of 24 min. As shown in Fig. 3A, the responses of the atrium to CCh were rapid and were able to be repeated. CCh resulted in a transient decrease followed by an increase in ANP secretion (Fig. 3Aa), which was coincident with changes in ECF translocation (Fig. 3Ab), atrial stroke volume and pulse pressure (Fig. 3Ad and Ae). CCh increased atrial myocyte ANP release (Fig. 3Ac). The CCh-induced increases in ANP release were able to be repeated and the values observed at the first, second and third exposures were not significantly different from each other (Figs. 3Ac and 4A). The CCh-induced decreases in atrial dynamics were able to be repeated and the responses at the first, second and third exposures were not significantly different from each other (Figs. 3Ad and Ae and 4B). In the absence of ISO, CCh decreased cAMP efflux concentration slightly, but the response was not prominent. Ratio changes over the control of cAMP efflux concentration induced by CCh were 0.779 ± 0.032, 0.770 ± 0.198, and 0.768 ± 0.071 at the first, second and third exposures, respectively ($n=4$).

Does adrenoceptor stimulation decrease ANP release and affect CCh-induced ANP release?

The effect of ISO on ANP release, and the effect of repetition of CCh administration in the presence of ISO were tested. As shown in Fig. 5A, ISO decreased ANP secretion (Fig. 5Aa) and increased stroke volume and pulse pressure (Fig. 5Ad and Ae). ISO decreased ANP release by 46.13 ± 5.30%, which was significantly different from the values obtained during the corresponding period in the vehicle-infused group (11.80 ± 3.96%, $p<0.001$; Figs. 3Ac, 4A and 5Ac). ISO increased cAMP efflux concentration significantly (Fig. 7B). In the presence of ISO, CCh resulted in a transient decrease, followed by an increase, in ANP secretion (Fig. 5Aa), which was coincident with changes in ECF translocation, and stroke volume and pulse pressure (Fig. 5Ab, Ad and Ae). In the presence of ISO, CCh increased ANP release concomitantly with decreases in atrial dynamics and cAMP efflux concentration (Figs. 4A and B, 5Ac and 7B). Ratio changes over the control of cAMP efflux concentration induced by CCh were 0.585 ± 0.046, 0.610 ± 0.035, and 0.588 ± 0.039 at the first, second, and third exposures, respectively. The responses to CCh were rapid and were able to be repeated. In the presence of ISO, the CCh-induced increases in ANP release and decreases in atrial pulse pressure were able to be repeated, and the responses were not significantly different from each other (Fig. 4A and B). The effects of ACh (3.0 μM) were very similar to those of CCh, in terms of ANP release and atrial dynamics. ACh increased ANP release by 39.25 ± 9.06%, 89.57 ± 5.49%, and 77.11 ± 20.34%, and ACh decreased pulse pressure by 51.09 ± 2.45%, 54.52 ± 3.01%, and 54.48 ± 3.15% at the first, second, and third exposures, respectively ($n=3$). These findings demonstrated that stimulation of β-adrenoceptor decreased basal levels of ANP release, and activation of mAChR increased ANP release, concomitantly with decreases in cAMP efflux and atrial dynamics in both the presence and absence of ISO. Subsequent experiments were performed in the presence of ISO.

Are the effects of muscarinic stimulation with CCh on ANP release mediated by M_2 mAChR?

To define the receptor involved in the CCh-induced increase in ANP release, selective M_2 mAChR antagonists were tested. Methoctramine attenuated CCh-induced changes in ANP release and pulse pressure in a concentration-dependent manner (Fig. 6A and B). Similarly, but more potently, AQ-RA 741, a selective M_2 mAChR antagonist which is structurally different to methoctramine, significantly attenuated the CCh-induced increase in ANP release and decrease in pulse pressure. These findings demonstrated that both the CCh-induced increase in ANP release and decrease in atrial dynamics were caused by an activation of M_2 mAChR.

Are the effects of muscarinic stimulation with CCh on ANP release mediated by K^+_{ACh} channels?

Both the K^+_{ACh} channel activation by direct Gβγ-channel interaction and the decrease in cAMP levels by Gαi-adenylyl cyclase pathway are known to be downstream signaling components of M_2 mAChR activation (Brodde and Michel, 1999; Yamada et al., 1998). To define the role of G-protein-gated K^+_{ACh} channels for the CCh-induced increase in ANP release, tertiapin was used. As shown in Fig. 3B,

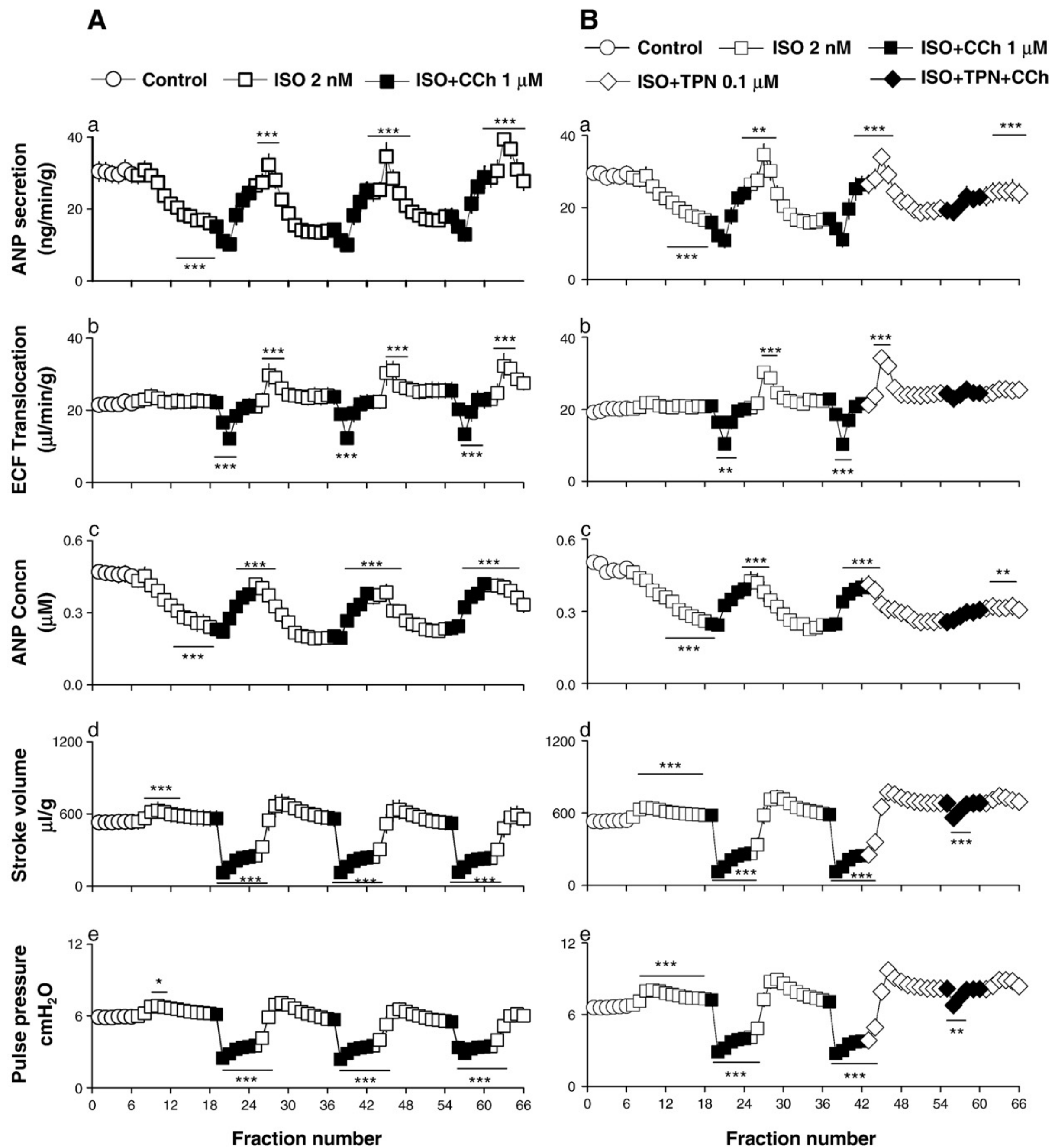

Fig. 5. Effects of carbachol and its modulation by tertiapin on ANP release and atrial dynamics in the presence of isoproterenol in beating rabbit atria. (A) effects of carbachol (CCh) on ANP secretion (a), ECF translocation (b), ANP concentration (c), stroke volume (d), and pulse pressure (e) (n=9). (B) modulation by tertiapin (TPN) of the CCh-induced changes in ANP secretion (a), ECF translocation (b), ANP concentration (c), stroke volume (d), and pulse pressure (e) (n=8). **p<0.01, ***p<0.001 vs. the values before the addition of ISO or CCh.

tertiapin had no significant effect on the basal levels of ANP release and atrial dynamics. Tertiapin blocked CCh-induced increases in ANP secretion and myocyte ANP release and decreases in ECF translocation (Fig. 3Ba and Bc), and partially attenuated atrial dynamics (Fig. 3Be). Similarly, tertiapin blocked CCh-induced changes in ANP secretion, myocyte ANP release, and ECF translocation (Fig. 5Ba and Bc), and partially attenuated atrial dynamics in the presence of ISO (Fig. 5Bd and Be). Tertiapin attenuated CCh-induced changes in ANP release and atrial dynamics in a concentration-dependent manner (Fig. 7A). Tertiapin (0.6 μM) almost completely blocked the CCh-induced changes of ANP release and atrial dynamics. As shown in Fig. 7B, atrial cAMP efflux levels, another downstream signaling component of M_2 mAChR activation, were significantly decreased by CCh. The CCh-induced decrease in cAMP levels was not significantly changed by tertiapin.

Are the effects of CCh related to the PTX-sensitive G-proteins?

Next, to evaluate the role of PTX-sensitive G-proteins, PTX was administered to rabbits. Basal levels of ANP release and atrial dynamics were not significantly different between atria from control and PTX-pretreated rabbits. The CCh-induced increase in ANP release and decrease in atrial dynamics were significantly inhibited in the atria from PTX-pretreated rabbits [mean±SE for the first two exposures; increase in ANP release: 13.43±6.23%, n=9, vs. 76.60±11.06, n=3, sham-treated (82.52±23.64%, n=9, control), p<0.001;

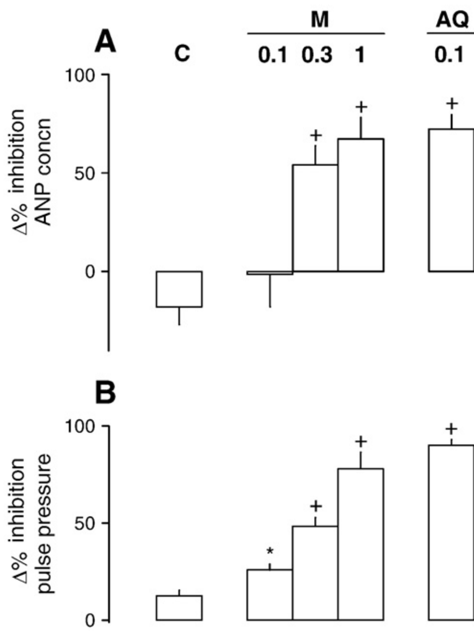

Fig. 6. Inhibition by M_2 muscarinic receptor antagonists of the CCh-induced changes in the presence of isoproterenol. (A) inhibition by methoctramine and AQ-RA 741 of the CCh-induced increase in ANP concentration. (B) inhibition by methoctramine and AQ-RA 741 of the CCh-induced decrease in pulse pressure. C, control (n=9); M, methoctramine; 0.1 μM (n=2), 0.3 μM (n=3), 1.0 μM (n=3); AQ, AQ-RA 741, 0.1 μM (n=5). *p<0.05, +p<0.001 vs. control (C). Data were expressed as percent differences over the control second exposure of CCh (in Materials and methods). Second exposure of CCh in the presence of vehicle was used as control and the third exposure of CCh in the presence of vehicle or modulator was used as control (C) or experimental (M, AQ).

decrease in pulse pressure: −0.87±2.68%, n=9, vs. −51.10±3.01, n=3, sham-treated (−59.62±3.29%, n=9, control), p<0.001].

These findings demonstrated that G-protein-gated K^+_{ACh} channel activation, but not the Gαi-coupled decrease in cAMP levels, was involved in the CCh-induced increase in atrial myocyte ANP release and decrease in atrial dynamics.

Are the effects of CCh related to L-type Ca^{2+} channel activity?

To define the role of Ca^{2+} entry via L-type Ca^{2+} channels in the CCh-induced increase in ANP release, CCh was introduced in the presence of nifedipine. The CCh-induced increase in ANP release was attenuated in the presence of nifedipine (25.46±3.21%, n=6, in the presence of nifedipine, vs. 107.51±31.37%, n=5, in the absence of nifedipine, p<0.05). Similarly, the CCh-induced decrease in pulse pressure was attenuated in the presence of nifedipine (−20.63±3.07%, n=6, in the presence of nifedipine, vs. −41.36±1.94%, n=5, in the absence of nifedipine, p<0.001). These findings demonstrated that intact L-type Ca^{2+} channel activity was required for the CCh-induced changes in ANP release and atrial dynamics.

Discussion

The present study showed for the first time that the G-protein-gated muscarinic K^+ channel, the K^+_{ACh} channel, is involved in the regulation of atrial myocyte ANP release in beating rabbit atria. Stimulation of M_2 mAChR with muscarinic agonist increased ANP release via K^+_{ACh} channel activation. The present data also showed an opposing role of adrenoceptors and mAChR for the regulation of atrial ANP release.

In the present study, the response of ANP release to the activation of mAChR was accompanied by changes in atrial volume. Therefore, it is possible that the mechanisms responsible for the increase in ANP release involve two pathways: a direct effect from stimulation of mAChR, and an indirect effect following changes in atrial volume. It had been shown in a previous study that ANP release is dissociated from changes in atrial dynamics (Kuroski-De Bold and De Bold, 1991). Also, in our previous experiments, we showed that basal ANP release is positively controlled by changes in atrial volume and pacing frequency, and also negatively modulated by second messengers, including cAMP, cGMP, and Ca^{2+} (Cui et al., 2002; Wen et al., 2000, 2004). The modulation of ANP release was not always in parallel with the changes in atrial dynamics (Cui et al., 2002; Wen et al., 2000, 2004). Furthermore, as shown in the present study, the CCh-induced increase in ANP release was dissociated from the decrease in atrial dynamics in the later stages of the treatment (Fig. 2A) and at lower concentrations of the agent (Fig. 2B and C), and in the presence of tertiapin (Figs. 3B and 5B). Taken together, these findings suggest that both the CCh- and ACh-induced increase in ANP release may be directly related to the stimulation of mAChR.

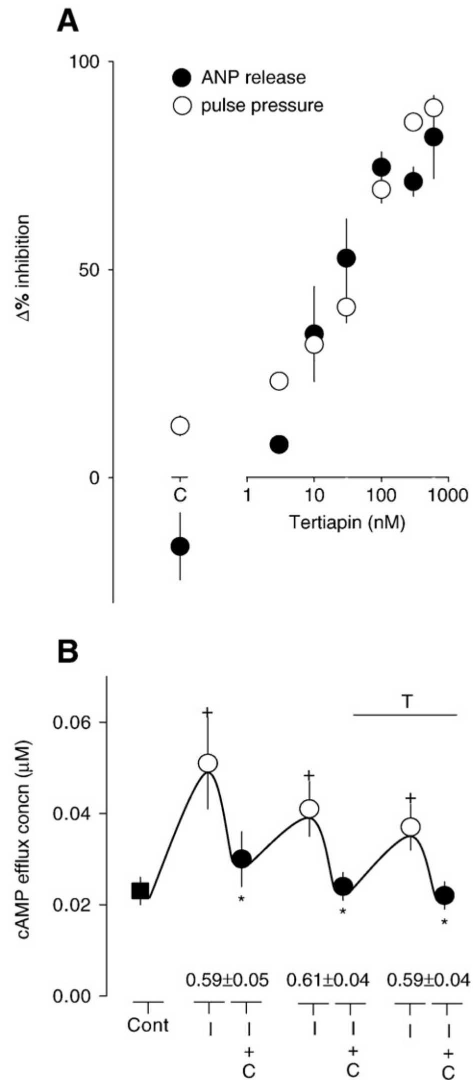

Fig. 7. Effects of tertiapin on the CCh-induced increase in ANP release and decreases in pulse pressure and cAMP efflux levels in the presence of isoproterenol in beating rabbit atria. (A) inhibition by tertiapin of the CCh-induced increase in ANP release and decrease in pulse pressure. C, control (n=9); tertiapin 3 nM (n=2), 10 nM (n=7), 30 nM (n=8), 100 nM (n=8), 300 nM (n=8), 600 nM (n=7). Data were expressed as in Fig. 6. C, control. (B) effects of tertiapin on CCh-induced decrease in cAMP efflux levels (pooled number of experiments (n=15); tertiapin 600 nM (n=3), 300 nM (n=2), 100 nM (n=1), 30 nM (n=3), 10 nM (n=3), 0 nM, control (n=3). Ratio changes in cAMP efflux concentration by CCh were 0.585±0.046, 0.610±0.035, and 0.588±0.039 at first, second and third exposures, respectively. Cont, control; T, tertiapin; I, isoproterenol (2 nM); C, carbachol (1.0 μM). +p<0.01 vs. control (Cont); *p<0.001 vs. isoproterenol (I).

M_2 mAChR-Gβγ-K^+_{ACh} channel signaling for ANP release

The CCh-induced increase in ANP release and decreases in atrial stroke volume and intra-atrial pulse pressure were blocked by M_2 mAChR antagonists. This finding is consistent with the report that the cardiac mAChR is predominantly of the M_2 subtype (Brodde and Michel, 1999). To define the downstream signaling of M_2 mAChR activation, we tested whether the Gβγ-gated K^+_{ACh} channel (Yamada et al., 1998) is involved. Tertiapin is a potent and selective blocker of G-protein-gated K^+_{ACh} channels (Jin and Lu, 1998, 1999), which are preferentially expressed in the cardiac atria (Dobrzynski et al., 2001). Tertiapin has been found to be a selective and potent blocker of the K^+_{ACh} channel in cardiac atrial myocytes (Drici et al., 2000; Kitamura et al., 2000). In the present study, tertiapin blocked the CCh-induced increase in ANP release and attenuated the decrease in atrial dynamics in a concentration-dependent manner. The half-maximum inhibitory concentration of tertiapin was less than 30 nM, which was in agreement with previous reports showing the decrease in the I_{KACh} current in rabbit (Kitamura et al., 2000) and rat (Drici et al., 2000) atrial cardiomyocytes and transfected cultured cells (Jin and Lu, 1999).

The decrease in cAMP levels by CCh shown in the present study was expected, because M_2 mAChR activation by CCh is coupled to Gαi, another downstream signaling component, which is inhibitory to adenylyl cyclase activity. Although previous reports showed that an increase in cAMP levels resulted in a decrease (Cui et al., 2002; Ruskoaho et al., 1990) or increase (Rukoaho et al., 1986) in atrial ANP release, the CCh-induced decrease in cAMP levels is not related to the CCh-induced increase in ANP release over a short duration, as used in the present experimental protocols. Tertiapin had no significant effect on the CCh-induced decrease in cAMP levels. These findings indicate that the CCh-induced changes are not related to the adenylyl cyclase-cAMP signaling pathway. Furthermore, this demonstrates that K^+_{ACh} channel activation results in an increase in atrial ANP release and a decrease in atrial dynamics.

In the present study, the CCh-induced changes in ANP release and atrial dynamics were not observed in the atria from rabbits pretreated with PTX, and this suggests that the CCh-induced changes have a close association with the PTX-sensitive G-proteins. This finding is consistent with the reports that PTX-sensitive G-proteins are involved in the activation of K^+_{ACh} channels via the M_2 mAChR (Yamada et al., 1998). Therefore, these results demonstrate that the CCh-induced increase in ANP release is caused mainly by activation of G-protein-gated K^+_{ACh} channels via the M_2 mAChR. This notion is also consistent with the previous report that the mAChR complex controls the K^+_{ACh} channels directly (Soejima and Noma, 1984).

Because atrial K^+_{ACh} channel activation is accompanied by shortening of the action potential, this may result in a decrease in intracellular Ca^{2+}, and thus contractility (Dhein et al., 2001), and also an increase in ANP release (Fig. 8). Previously, it was shown that Ca^{2+} is a negative regulator for atrial ANP release (De Bold and De Bold, 1989; Ito et al., 1988; Ruskoaho et al., 1990; Wen et al., 2000). In the present study, the CCh-induced increase in ANP release and decrease in atrial dynamics were attenuated by inhibition of L-type Ca^{2+} currents. This finding indicates that intact Ca^{2+} entry via L-type Ca^{2+} channels (the negative regulator) is a prerequisite for the CCh-induced increase in ANP release (Fig. 8).

Inward-rectifier K^+ channels are involved in various biological functions. For example, the G-protein-gated muscarinic K^+_{ACh} channel controls cardiac rate and conduction. As shown in the present study, the K^+_{ACh} channel also has a role in the regulation of cardiac secretory function.

It is known that plasma ANP levels are increased in patients with atrial fibrillation (Roy et al., 1987). However, the mechanism responsible for this finding remains to be defined. The present data suggest that the K^+_{ACh} channel-related increase in atrial myocyte ANP release may, at least in part, be responsible for the increased levels of plasma ANP observed in patients with atrial fibrillation. Previously, it was shown that stimulation of atrial mAChRs by vagal stimulation (Euler and Scanlon, 1987) and enhanced vagal tone (Chen et al., 1998) could give rise to atrial fibrillation. Also, recently, G-protein-gated K^+_{ACh} channel activation has been shown to be associated with atrial fibrillation (Dobrev et al., 2005; Hashimoto et al., 2006). In certain patients with atrial fibrillation, K^+_{ACh} channels were found to develop agonist-independent constitutive activity which did not require the presence of receptor agonist in order to be activated (Dobrev et al., 2005). These patients would be expected to show high levels of plasma ANP.

Opposing roles of adrenoceptors and mAChRs for the regulation of ANP release

ISO decreased atrial myocyte ANP release concomitantly with an increase in cAMP efflux levels, stroke volume and intra-atrial pulse pressure. By contrast, CCh and ACh increased atrial ANP release concomitantly with a decrease in atrial dynamics in a concentration-dependent manner. The CCh-induced increase in ANP release and decreases in atrial dynamics and cAMP efflux in the presence and absence of ISO were able to be repeated. CCh increased ANP release for about 12 min, and thereafter the levels started to decline toward the

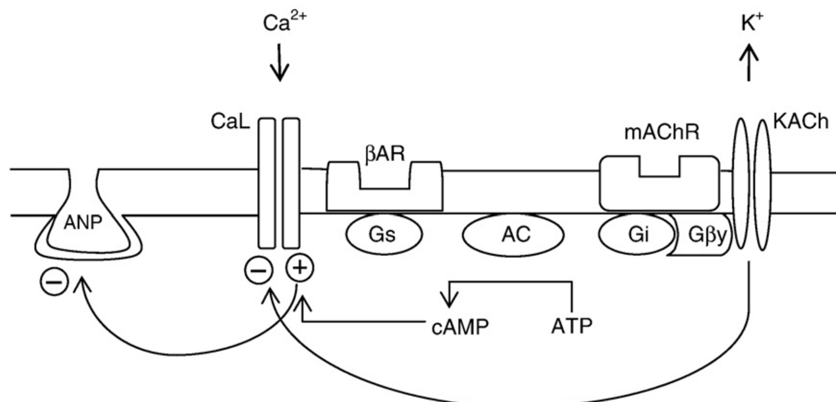

Fig. 8. Schematic diagram showing the mechanism by which carbachol increases atrial myocytic ANP release. Carbachol increases ANP release via M_2 muscarinic ACh receptor-K^+_{ACh} channel signaling. M_2 mAChR stimulation activates K^+_{ACh} channels via G-protein βγ. Hyperpolarization induced by K^+_{ACh} activation may shortens the duration of L-type Ca^{2+} channel depolarization and then decreases the Ca^{2+} entry via the channels. Here, the Ca^{2+} entry is a negative regulator for ANP release. The decrease of Ca^{2+} entry may relieve the tonic inhibition by Ca^{2+} of the atrial myocyte ANP release (deinhibition). The decrease of Ca^{2+} entry also attributes to the negative inotropic effect of CCh. K_{ACh}, G-protein-gated muscarinc K^+_{ACh} channels; mAChR, M_2 muscarinic ACh receptors; βAR, β-adrenergic receptors; CaL, L-type Ca^{2+} channels; ANP, ANP granules; Gs, G-protein stimulatory α; Gi, G-protein inhibitory α; Gβγ, G-protein βγ; AC, adenylyl cyclase; +, activation; −, inhibition.

control values. This may have been due to desensitization of the G-protein-gated K^{+}_{ACh} channels. The present finding of an increase in ANP release in response to activation of mAChRs is consistent with some previous reports (Inoue et al., 1988; Ruskoaho et al., 1985; Sonnenberg and Veress, 1984), but is in contrast with another (Schiebinger et al., 1987). Also, the finding of an ISO-induced decrease in ANP release concomitantly with an increase in cAMP levels is consistent with the previous reports (Cui et al., 2002; Ruskoaho et al., 1990). Previously, it was shown that cAMP is a negative regulator for ANP release (Cui et al., 2002; Ruskoaho et al., 1990). However, this finding is in contrast with reports showing ISO to cause an increase in ANP release (Schiebinger et al., 1987).

The reason for this difference may be related to the methodology. In the present study, using an atrial preparation, it was possible to measure the changes in ECF translocation, along with atrial volume and intra-atrial pulse pressure. In addition, the beating preparation efficiently controls the extracellular environment of the atrial myocytes in response to beat-by-beat changes in atrial volume. Moreover, there have been reports that ANP release is controlled by changes in atrial volume (Cho et al., 1995; Dietz, 1984; Lang et al., 1985). As shown previously (Cho et al., 1995), stretch-activated ANP secretion is controlled by a two-step sequential mechanism: myocyte release of ANP into the surrounding paracellular space followed by the translocation of ECF with the released ANP into the bloodstream. The first step, myocyte ANP release, is inversely related to the intracellular Ca^{2+} concentration (Cui et al., 2002; Wen et al., 2000), while the second step, ECF translocation, is positively controlled by atrial contraction (Cho et al., 1995). Atrial workload determines the translocation of the ECF and released ANP, with waning of the translocation at higher myocardial workloads (Cho et al., 1995; Wen et al., 2000). Therefore, agents which give rise to a positive inotropic effect through an increase in intracellular Ca^{2+} may have complex actions on ANP secretion: a decrease in myocyte ANP release through increased Ca^{2+} levels and also a slight increase in the translocation of ECF and released ANP by elevated atrial workload. In this case, ANP secretion may be decreased. In addition, the differences in the effects of various agents might be due to the basal atrial workload; species differences may also be involved.

The present study suggests that the autonomic nervous system is involved in the regulation of body fluid homeostasis via cardiac hormone release, which is implicated both in physiological adaptation to the environment and also in pathological derangements.

Conclusion

The present study shows that G-protein-gated K^{+}_{ACh} channels are involved in the regulation of atrial myocyte ANP release. The present findings also demonstrate that mAChRs and adrenoceptors have opposing roles in the regulation of atrial ANP release.

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