Interaction of Adenosine and Acetylcholine on the Bullfrog Atrium

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Abstract As adenosine has a potent stabilizing action on catecholamine stimulation in the myocardium, the mode of interaction of adenosine and acetylcholine (ACh) was studied with regard to the membrane potential, current and tension components of the bullfrog atrium, using the single or double-sucrose gap method. Adenosine (10^{-4} – 3×10^{-3} M) augmented the twitch contraction in the presence of ACh (10^{-9} – 5×10^{-7} M) by lengthening the duration of the action potential. The dose-tension response curve for ACh was modified by adenosine, producing a rise of the inhibitory threshold of ACh, and the modification showed a non competitive interaction of these compounds. Under the voltage clamp, ACh-induced steady current (I_{ACh}) was inhibited by adenosine non-competitively. The known inhibition of slow inward current (I_s) by ACh was enhanced by adenosine, while the delayed outward current (I_x) was markedly suppressed. I_s -dependent and -independent tension components were both inhibited by adenosine, thereby suggesting a decrease in intracellular concentrations of calcium. The potent suppression of I_{ACh} and I_x induced by adenosine, however, appeared to mitigate the inhibitory action of ACh on the action potential and twitch contraction.

Since the first report by DRURY and SZENT-GYÖRGYI (1929), the inhibitory actions of adenine compounds on the heart have been well investigated (see DRUMMOND and SEVERSON, 1979; BURNSTOCK, 1980). These studies have shown that adenosine and adenine nucleotides have an overall effect similar to that of acetylcholine (ACh) by slowing and weakening the heartbeat. In the mammalian atrium, the depression of contraction with adenosine is accompanied by a shortening of the action potential, possibly due to an increased potassium permeability (JOHNSON and MCKINNON, 1956; HOLLANDER and WEBB, 1957), and in the frog sinus venosus, adenosine and related compounds produce a marked hyperpolarization presumably reflecting an increase in potassium conductance (HARTZELL, 1979). SCHRADER *et al.* (1975) and BELARDINELLI *et al.* (1979), however, reported

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that in guinea-pig and rat atria, adenosine is a potent inhibitor of the catecholamine-induced slow action potential and they suggested a possible inhibitory action on calcium influx. GOTO *et al.* (1977, 1978, 1979) examined the effects of adenine compounds on the bullfrog atrium under voltage clamp and found that energy-poor nucleotides and adenosine inhibited the slow inward current and the phasic and tonic tension components.

Thus, the actions of adenosine seem to resemble those of ACh which is known to increase the potassium permeability (TRAUTWEIN *et al.*, 1956; HUTTER, 1961; GARNIER *et al.*, 1978) and to reduce the slow inward current (IKEMOTO and GOTO, 1975, 1977; GILES and NOBLE, 1976). In the present study which deals with possible additive or antagonistic interactions of the effects of ACh and adenosine on the bullfrog atrium, we attempt to clarify the role of adenosine in the cholinergic action on the myocardium in contrast to the potent stabilizing action of adenosine on catecholamine stimulation (SCHRADER *et al.*, 1975, 1977; GOTO *et al.*, 1978).

METHODS

Preparation and experimental apparatus were the same as reported previously (GOTO *et al.*, 1978, 1979). In brief, thin muscle bundles (0.4–0.5 mm in diameter and 5–6 mm in length) were isolated from the left atrium of the bullfrog, *Rana catesbeiana*, and by means of a conventional double sucrose-gap method, the membrane potential, current, and tension of the muscle were measured simultaneously under voltage clamped and unclamped conditions.

Cumulative dose-tension response curves for ACh with or without adenosine (RESULTS 1) were determined using the single gap method (YATANI *et al.*, 1978). For these tests, the right side sucrose-gap was omitted by removing the rubber membranes, and the large terminal compartment was used as the test chamber.

ACh-induced extra current (I_{ACh}) was measured by the method of GARNIER *et al.* (1978), as described in the text (RESULTS 3), and the voltage dependency of I_{ACh} was estimated from the difference in the terminal current level of the voltage-current relationships obtained with 2.0 sec pulses before and after application of ACh.

All experiments were performed after an equilibration time of over 60 min in normal Ringer's solution and at a constant temperature of $17 \pm 0.5^\circ\text{C}$.

RESULTS

1. Effects of adenosine on the dose-tension response curve for ACh

To examine the general mode of interaction between adenosine and ACh on the tension response, dose-tension response curves for ACh were first determined in the presence and absence of adenosine (10^{-4} – 3×10^{-3} M). Figure 1 shows the results of cumulative dose-response relations in the bullfrog atrium. ACh pro-

Fig. 1. Cumulative dose-tension response curve for ACh (●), and the curves modified in the presence of atropine (■, 5×10^{-9} M; ▲, 10^{-6} M) or adenosine (○, 3×10^{-8} M). Values are means of five experiments ($n=5-3$) with or without standard error. Note a non-competitive inhibition by adenosine of the depressive effects of ACh in comparison with a competitive inhibition by atropine.

duced a dose-dependent inhibition of the twitch contraction, showing a half-maximal inhibition (pD_2) at about 7×10^{-8} M with a threshold at around 10^{-9} M. The contraction all but disappeared with concentrations of ACh over 10^{-6} M.

In the presence of adenosine (3×10^{-8} M), the dose-tension response curve for ACh was modified, showing a higher threshold of inhibition (3×10^{-8} M) and a steep fall of twitch tension with increases in the concentration of ACh. Thus, a significant increase in tension by addition of adenosine was observed when the concentrations of ACh were between 10^{-9} – 10^{-7} M, suggesting an antagonistic action between ACh and adenosine. The mode of antagonistic action differed from that seen with atropine which produced a typical parallel shift of the dose-response curve. The interaction between ACh and adenosine appeared to be non-competitive.

Figure 2 shows an example of the interaction between the effects of ACh and adenosine on the membrane potential and twitch contraction. The sole action of adenosine was a slight depolarization of the membrane and a prolongation of the action potential accompanied by a suppression of twitch contraction (GOTO *et al.*, 1978). In the presence of ACh (10^{-8} – 10^{-7}), however, the addition of adenosine (3×10^{-8} M) produced a considerable prolongation of the action potential and an augmentation of the twitch tension (Fig. 2A). Adenosine 10^{-4} M produced similar effects (Fig. 2B). Neither the resting gap potential nor the overshoot of gap action potential changed appreciably. Thus, when application and removal of adenosine were repeated in the constant presence of ACh, the tension response always appeared to be larger during the period of application.

2. Effects of adenosine in the presence of ACh on the membrane currents and tension components

In the myocardium, ACh induces, 1) an increase in K permeability (TRAU-

Fig. 2. Interaction between the effects of ACh and adenosine on the membrane potential (V) and contractile tension (T). A) ACh (5×10^{-8} M) produced a shortening of action potential and a decrease of contractile tension. Addition of adenosine (3×10^{-8} M) in the presence of ACh elicited a prolongation of action potential and an enhancement of contraction. B) Similar data with a lower concentration of adenosine. a, superimposed records of control and response 4 min after ACh (5×10^{-8} M, $\bullet$); b, those of 2 min ($\blacksquare$) and 5 min ($\blacktriangle$) after addition of adenosine (10^{-4} M), and 15 min after washing with normal Ringer. Vertical bars are calibrations of 100 mV, and 0.02 g for voltage and tension, respectively. Horizontal bars are time calibrations of 1.0 sec and 1 min for A, and 1.0 sec for B.

Fig. 3. Effects of ACh, and ACh plus adenosine on the membrane currents and tension components. A) Control responses to small (60 mV, 0.2 sec) and large depolarizing pulses (140 mV, 2.0 sec) and responses 4 min after ACh (5×10^{-8} M, white dots) are superimposed. B) Similar superimposed records of responses in the presence of ACh (white dots) and those 5 min after further addition of adenosine (3×10^{-8} M, white squares). Vertical bars from the top to the bottom are calibrations of 2 μ A, 100 mV and 0.1 g for membrane current (I), voltage (V) and tension (T), respectively. Horizontal bar marks time, 2 sec.

TWEIN *et al.*, 1956; HUTTER, 1961), 2) a decrease of the slow inward current (I_s) (IKEMOTO and GOTO, 1975; GILES and NOBLE, 1976; GARNIER *et al.*, 1978), and 3) an inhibition of I_s -dependent phasic tension and an augmentation of I_s -independent tonic tension (IKEMOTO and GOTO, 1975). Most of these effects were confirmed in the present experiment. Figure 3A shows the effects of ACh on the I_s -dependent and -independent tension responses to small (60 mV, 0.2 sec) and large (140 mV,

2.0 sec) depolarizing pulses in the constant presence of TTX (1.5×10^{-6} M), respectively, in which I_s became nearly maximum in the former and negligible in the latter. ACh (5×10^{-8} M, white dots) inhibited the I_s and I_s -dependent tension and augmented the I_s -independent tension. When adenosine (3×10^{-3} M) was added in the presence of ACh (Fig. 3B), the I_s and I_s -dependent tension for small pulses were further diminished and the outward current as well as the I_s -independent tension for large pulses was markedly inhibited. The inhibition of the outward current appeared both in the instantaneous and time-dependent current components.

Next, the membrane current-voltage relation were examined to assess in detail the mode of interference. Figure 4A shows the effects of ACh, and 4B, those of adenosine in the presence of ACh. TTX (1.5×10^{-6} M) was constantly present in the bathing solution. ACh (5×10^{-7} M) inhibited the I_s , produced a so-called ACh extra current (I_{ACh} , GARNIER *et al.*, 1978) and increased the amplitude of terminal currents for hyperpolarizing and depolarizing pulses (Fig. 4A). When adenosine (3×10^{-3} M) was added in the presence of ACh (Fig. 4B), there was a marked depression of the terminal current levels and the I_s diminished further. The depression of the terminal current, however, was more conspicuous with stronger

Fig. 4. Current-voltage relations in TTX Ringer, and effects of ACh and ACh plus adenosine on the membrane currents. A) The relationships before ($\circ$, $\square$) and 8–14 min after ACh (5×10^{-7} M, $\bullet$, $\blacksquare$). B) Those with ACh ($\bullet$, $\blacksquare$) and 10–15 min after addition of adenosine (3×10^{-3} M, $\circ$, $\square$). Circles denote peak value of inwardly directed current, and squares, terminal current levels at the end of 2.0 sec pulses. Note the appearance of ACh-induced extra current (I_{ACh}) and a decrease of I_s in Fig. A, and a marked inhibition of I_{ACh} , I_s , I_x by further addition of adenosine in Fig. B.

pulses of depolarization, suggesting that the effect was not due only to the elimination of the I_{ACh} which shows an anomalous rectification (GARNIER *et al.*, 1978; NOMA and TRAUTWEIN, 1978; NOMA *et al.*, 1979), but was largely caused by an inhibition of the delayed outward current (I_x). These results indicate that the prolongation of the action potential and the partial recovery of tension after adenosine in the presence of ACh are mainly due to a potent inhibition of these outward currents (I_{ACh} , I_x).

3. Antagonistic effects of adenosine on the ACh-induced extra current

Since the antagonistic interaction between adenosine and ACh mainly occurred in the terminal current levels, the interaction was examined on the isolated ACh-induced extra current (I_{ACh}). The I_{ACh} was measured following the method of GARNIER *et al.* (1978). The experimental protocol is shown in Fig. 5A; *i. e.*, the membrane was maintained at a given depolarized potential (+50 or +60 mV from the resting potential) and ACh was introduced in steps of log-unit concentrations, at 5 min intervals. ACh produced an outward current which reached a maximum value corresponding to the concentration in 1 min, and the total amount of I_{ACh} extra current increased with additional concentration of ACh. After the addition of adenosine (10^{-3} M), however, I_{ACh} extra current was markedly inhibited, as shown in Fig. 5B.

The results of these and similar experiments were plotted as dose- I_{ACh} response curves in Fig. 5C. The I_{ACh} appeared with a threshold of 10^{-7} M or less, increased dose-dependently and became saturated at around 10^{-4} M. pD_2 was about 2×10^{-6} M. After the addition of adenosine (10^{-3} M), the I_{ACh} was strongly inhibited, but the inhibition of the maximal response was greater in the case of +60 mV depolarization than that of +50 mV, thereby suggesting anomalous characteristics of the background current. The threshold and relative dose-dependency were not appreciably altered. Higher concentrations of adenosine (3×10^{-3} M) eliminated the I_{ACh} (figure not shown). The dose-dependent effects of adenosine indicate that the interaction between ACh and adenosine on I_{ACh} is non-competitive.

4. Action of adenosine in the presence of a low concentration of acetylcholine

Adenosine produced non-competitive inhibition of I_{ACh} . This inhibition, however, would hardly explain the modification of the dose-response curve due to adenosine (see Fig. 1), because with the application of adenosine, the tension was recovered when the concentrations of ACh were below 10^{-7} M where I_{ACh} , and hence, the inhibition of I_{ACh} by adenosine, were rarely observed (Fig. 5C).

Another explanation for the modification may be the recovery of the I_s due to adenosine which heretofore had been inhibited by ACh. The possibility was examined on I_s isolated in Ringer solution containing TTX (1.5×10^{-6} M), but such was not the case. Figure 6A shows the effects of ACh (5×10^{-8} M) on the voltage-

Fig. 5. Isolated I_{ACh} and effect of adenosine. The membrane potential was held at a depolarized level of 50 mV, and ACh was introduced cumulatively in steps of log-unit concentration. A) I_{ACh} in control condition. B) Depression of I_{ACh} in the presence of adenosine (10^{-8} M). Vertical bars from the top to the bottom are calibration of 0.5 μ A, 100 mV and 0.2 g for current (I), voltage (V) and tension (T). Horizontal bars are time calibrations of 5 min, 2 min and 1 sec for the slow, middle and fast records, respectively. C) Dose- I_{ACh} response curves in control conditions (●, ■) and the effects of adenosine (10^{-8} M, ○, □). Squares are data at 50 mV depolarization (H. P.) and circles, at 60 mV. Adenosine markedly inhibited I_{ACh} .

Fig. 6. Effects of low concentration of ACh (5×10^{-8} M) and that plus adenosine (3×10^{-3} M) on the current-voltage relationships in the presence of TTX (1.5×10^{-6} M) and atropine (10^{-9} M). A) Relationships before ($\circ$, $\square$) and 9–15 min after ACh ($\bullet$, $\blacksquare$). B) Those after ACh ($\bullet$, $\blacksquare$) and 8–15 min after addition of adenosine ($\circ$, $\square$). Circles denote peak values of inwardly directed current and squares, terminal current levels at the end of 2.0 sec pulses. Note absence of I_{ACh} in A, and marked depression of I_s and I_x in B.

current relationship in the constant presence of TTX and atropine (10^{-9} M), and Fig. 6B, modification of the effects after further addition of adenosine (3×10^{-3} M). The low concentration of atropine was always used in order to eliminate endogenous release of ACh, particularly when the effects of low concentrations of ACh were examined. ACh with atropine in these concentrations did not elicit I_{ACh} , but did produce a distinct inhibition of I_s (Fig. 6A). Moreover, the inhibition was further enhanced after the addition of adenosine (Fig. 6B). The enhancement of inhibition was also observed with a higher concentration of ACh (5×10^{-7} M) (Fig. 4B). These results clearly ruled out the possibility of a recovery by adenosine of the inhibited I_s due to ACh.

On the other hand, it should be noted that the delayed outward current as well as the background current for hyperpolarization were markedly inhibited by the addition of adenosine (Fig. 6B). When the tail of the outward current (I_x) was isolated with long double step clamp pulses (Fig. 7A) and when the effects of ACh and adenosine on the maximally activated tail current of I_x were examined, we found that ACh (10^{-7} – 10^{-5} M) reduced the outward current tail, and that the addition of

Fig. 7. Effects of ACh and addition of adenosine on delayed outward current. A) Activation of delayed outward current. Grade of activation was observed on the tail current. Two step clamp pulses were used. 1st step pulses were depolarizations of 100 mV and of different durations (0–3 sec) from holding potential of -80 mV, and 2nd step pulses, constant depolarization of 50 mV and 10 sec. The maximal activation of tail current was obtained by 1st step pulse of 3.0 sec. B) The effects of ACh and adenosine on the maximally activated tail current. Amplitude of the tail current is estimated by the difference of the currents with or without 1st step pulse of 100 mV and 3 sec. a, control; b, 5 min after ACh (10^{-7} M) and c, 5 min after addition of adenosine (10^{-8} M). Note depression of delayed outward current during and after 1st step depolarization by ACh and further by addition of adenosine. In Fig. A, vertical bars from the top to the bottom are calibrations of 100 mV, 1 μ A and 0.02 g for membrane voltage (V), current (I), and tension (T), respectively. In Fig. B, calibrations of membrane current, 5 μ A, and voltage, 100 mV. Horizontal bars denote 5 sec in Fig. A and 10 sec in Fig. B. C) Dose-dependent inhibition of isolated delayed outward current by adenosine in the presence ($\bullet$) of ACh (10^{-7} M). Peak tail current after the 1st pulse was plotted against adenosine concentration.

adenosine further enhanced the depression (Fig. 7B). Reduction of the tail current due to ACh, however, did not necessarily indicate a depression of I_x since potassium accumulates under the action of ACh (GARNIER *et al.*, 1978) with even the possibility of relaxation of I_{ACh} (DiFRANCESCO *et al.*, 1980), although the threshold concentration for I_{ACh} was used in this case. The apparent depression due to further addition of adenosine is of significance, because if I_{ACh} was present the tail current would be enhanced *via* inhibition of I_{ACh} by adenosine. Thus, adenosine depresses the I_x in the presence of ACh, and such could be observed with adenosine in doses as low as 10^{-8} M (Fig. 7C).

DISCUSSION

In the bullfrog atrium, the twitch tension which was depressed by ACh in low concentrations was recovered by the application of adenosine. The dose-tension response curves for ACh (Fig. 1) and dose- I_{ACh} response curves (Fig. 5C) both revealed the presence of non-competitive interaction between ACh and adenosine. Although the nature of these interactions was not fully clarified, the presence of an antagonistic interaction contrasted with the potent stabilizing action of adenosine on catecholamine stimulation in both mammalian and amphibian myocardium (SCHRADER *et al.*, 1975, 1977; GOTO *et al.*, 1978, 1979). Thus, adenosine appears as a stabilizing agent not only for catecholamines but also for cholinergic stimulation.

The slow inward current (I_s) and ACh current (I_{ACh}). According to GLIES and NOBLE (1976), ACh in concentrations over 2.0×10^{-8} M reduced I_s in the bullfrog atrium, while relatively high doses of ACh (above 10^{-7} M) were required to increase the potassium permeability and decrease the membrane resistance. Contrarily, TEN EICK *et al.* (1976) reported that in mammalian atria, the concentration of ACh, which reduced the twitch tension to 30–40%, increased the steady-state outward current (I_{ACh}) without affecting the I_s and time-dependent outward current (I_x). In the guinea-pig papillary muscle, however, OCHI and HINO (1980) demonstrated that ACh depresses both the I_s and I_x without producing I_{ACh} . The discrepancy in these results strongly suggests that effects of ACh vary considerably with animal species and the portion of myocardium involved. In the present experiments on the bullfrog atrium, threshold concentration of ACh for the negative inotropic effect was about 10^{-9} M (Fig. 1), while that for the appearance of I_{ACh} was 10^{-7} M (Fig. 5). pD_2 value in the dose-tension response curve (7×10^{-8} M) was about 30 times lower than that in the dose- I_{ACh} response curve (compare Fig. 1 with Fig. 5C). Our results are in good accord with the observations of GILES and NOBLE (1976) and indicate that in the frog myocardium, the negative inotropic effect of ACh depends mainly on the inhibition of I_s .

Under these experimental conditions, further addition of adenosine produced a prolongation of action potential (Fig. 2) and a non-competitive augmentation of the twitch tension (Fig. 1) at low concentrations of ACh (10^{-9} – 10^{-7} M). The addition of adenosine, however, depressed the I_s and I_{ACh} (Fig. 6B, 5C). The depression of these currents would hardly explain the recovery of the twitch tension after adenosine. The remaining possibility is a depression of I_x , and we found the I_x to be extremely sensitive to adenosine, even in a concentration of 10^{-5} M. Thus, in the frog atrium, a depression of I_x appeared to play a principal role in the prolongation of the action potential by adenosine in the presence of ACh in low concentrations, although with higher concentrations, depression of I_{ACh} may participate, in the case of mammalian atrium (TEN EICK *et al.*, 1976). It is noteworthy that adenosine, which may enhance potassium permeability in the sinus node of frog heart (HARTZELL, 1979), strongly inhibits the potassium-related currents in the atrium.

Possible action of adenosine on cyclic nucleotides. Adenine nucleotides and adenosine evoke various cardiovascular responses, most of which involve changes in endogenous adenosine 3', 5' cyclic monophosphate (cAMP) and guanosine 3', 5' cyclic monophosphate (cGMP) (see DRUMMOND and SEVERSON, 1979; BURNSTOCK, 1980). In the isolated frog ventricle, the magnitude of twitch tension correlates with equivalent changes in the ratio of cAMP: cGMP, not only during augmentation of twitch contraction by isoproterenol (SINGH *et al.*, 1978) but also during hypodynamic depression (FLITNEY *et al.*, 1978) and during the action of adenosine and adenine nucleotides (SINGH and FLITNEY, 1980). Since ACh also increases cGMP, a nucleotide which may have regulatory actions antagonistic to those of cAMP in the myocardium (GEORGE *et al.*, 1973; see DRUMMOND and SEVERSON, 1979), the effect of the mode of interaction between ACh and adenosine on the contractile tension, membrane potential and current components should be considered in relation to alterations in the level of cyclic nucleotides.

A possible close relationship between the levels of cAMP and the slow inward current (I_s) has to be given attention, since intracellular administration of cAMP and application of membrane permeable mono- or di-butyryl cAMP both enhanced the I_s , the amplitude of plateau potential or of slow action potential in the partially depolarized myocardium (TSIEN *et al.*, 1972; TSIEN, 1973; REUTER, 1974; SCHNEIDER and SPERELAKIS, 1975). In preceding papers, we clarified the situation that adenosine depresses the I_s and I_s -dependent tension, particularly in the case of augmentation by adrenaline or caffeine (GOTO *et al.*, 1978, 1979). In the present experiments, adenosine diminished further the depressed I_s and I_s -dependent tension due to ACh (Figs. 3, 4). As our observations are in good parallel with the aforementioned principle, an explanation can be made on the bases.

Remaining to be determined are the relationships between cyclic nucleotides and outward currents such as I_{ACh} and I_x , since alteration of these currents did not always cause parallel alterations in I_s , and the ACh-dose dependency of these currents differed. Similarly, the I_s and I_x both decreased with application of adenosine in the presence of caffeine, but not in the presence of adrenaline where the I_s was mainly affected (GOTO *et al.*, 1978, 1979). One related factor is the alteration in the intracellular concentration of calcium ions, $[Ca]_i$ which would have a considerable effect on the potassium-related outward currents (ISENBERG, 1977). Adenosine generally diminishes Ca-fluxes, while caffeine increases these fluxes (GUTHRIE and NAYLER, 1967). We found that adenosine diminished the I_s , and I_s -dependent and -independent tensions in the presence of ACh, thereby strongly suggesting a decrease of $[Ca]_i$. This decrease of $[Ca]_i$ by adenosine may enhance the inhibition of I_x and the background current more than in the case of being solely due to a decrease in the levels of cAMP (SINGH and FLITNEY, 1980). ACh inhibits the I_s but possibly increases $[Ca]_i$ since it produces an increase in the potassium-induced contracture and the I_s -independent tonic tension (IKEMOTO and GOTO, 1977).

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