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ADENOSINE RECEPTORS IN FROG SINUS VENOSUS: SLOW INHIBITORY POTENTIALS PRODUCED BY ADENINE COMPOUNDS AND ACETYLCHOLINE

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SUMMARY

1. Membrane potential changes produced by adenosine and adenine nucleotides, acetylcholine, and vagus nerve stimulation were studied by intracellular recording in the sinus venosus of the frog, *Rana pipiens*.

2. Acetylcholine (ACh) released from the vagus nerve terminals evoked a slow hyperpolarization lasting several seconds in the cells of the sinus. Ionophoretic application of ACh from a micropipette produced a response which is similar in time course and amplitude to that evoked by vagus nerve stimulation. Bath application of ACh caused a steady hyperpolarization in quiescent preparations, or cessation of action potential generation in spontaneously active preparations.

3. Adenosine and adenine nucleotides produced hyperpolarizations when applied by addition to the bath or by ionophoresis from micropipettes. The hyperpolarization produced by ionophoresis of adenine compounds was somewhat slower than that produced by ACh.

4. Adenosine and the adenine nucleotides, 5′-AMP, 3′-AMP, 2′-AMP, and 5′-ATP were virtually equipotent in their action. Adenosine was at least 1000-fold more potent than other purine and pyrimidine nucleosides or adenine. Both the ribose and adenine groups were important for agonist activity.

5. The concentrations of agonist required to produce half-maximal responses were estimated from dose–response curves as 3×10^{-7} M for ACh and 2×10^{-6} M for ATP. ACh is about 7 times more potent than ATP in producing a hyperpolarization.

6. Adenine compounds act directly upon the cardiac muscle fibres: bath or ionophoretically applied adenine compounds act even when transmitter release from nerve terminals is blocked with high (Mn^{2+}) or when ACh receptors are blocked with atropine.

7. Adenine compounds act on the surface of the muscle fibre membrane. Analogues of adenosine which do not enter the cell are potent agonists of the receptor. An adenyl oligonucleotide too large to enter the cell was 2·6 times more potent per mole than adenosine in producing a hyperpolarization. Drugs such as dipyridamole and 6-(2-hydroxy 5-nitrobenzyl) thioguanosine, which are potent blockers of adenosine transport, potentiate the response of the sinus cells to adenosine.

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8. Aminophylline and theophylline are competitive antagonists of adenosine action. The apparent K_i for aminophylline inhibition was 5 μM.

9. The response produced by adenine compounds is partly caused by an increase in the permeability of the membrane to K^+. The maximum response to both ACh and adenine nucleotides approached the estimated level of E_K or E_{Cl}. Replacing extracellular chloride with impermeant isethionate had no effect on responses to ACh or adenine nucleotides. The hyperpolarization was not produced by an activation of an ouabain-sensitive pump since 20 μM-ouabain had little effect on the response to adenosine.

10. The response to vagus nerve stimulation is completely blocked by 50 nM-atropine. This suggests that under the conditions of this experiment virtually all of the response of sinus cells to nerve stimulation is accounted for by the action of ACh.

INTRODUCTION

The regulation of the heartbeat is commonly viewed in terms of the opposing influences of the automatic nervous system neurotransmitters, acetylcholine (ACh) and noradrenaline. ACh decreases the rate and strength of heartbeat by reducing the slow inward (Na^+/Ca^{2+}) current of the action potential (Ikemoto & Goto, 1975; Giles & Noble, 1976; Ten Eick, Nawrath, McDonald & Trautwein, 1976) and increasing an outward potassium current important in pace-maker activity (Noble, 1975; Garnier, Nargeot, Ojeda & Rougier, 1978). Noradrenaline has essentially opposite effects to those of ACh (reviewed in Noble, 1975).

In addition to the well studied effects of autonomic transmitters, a variety of other chemical substances have pronounced effects on cardiac rhythmicity. In particular, since the studies of Drury & Szent-Györgyi (1929), the inhibitory influences of adenine compounds on the heart have been described repeatedly (see references in Burnstock, 1972; Goto, Yatani & Tsuda, 1977). These studies have shown that adenosine and adenine nucleotides have an over-all effect similar to that of ACh: they slow and weaken the heartbeat. The cellular and membrane events underlying the actions of adenine compounds, however, are not well understood. Further, the physiological role of adenine compounds in regulation of the heartbeat remains to be elucidated.

The purpose of this investigation was to examine the response of the primary pace-maker region, the sinus venosus, of the frog heart to adenine compounds and to compare the response to that produced by ACh. This study reports that adenosine acts on a receptor located on the surface of sinus myocytes and, like ACh, causes a slow, long-lasting hyperpolarization of the membrane.

METHODS

Preparation. Experiments were performed at room temperature (19-25 °C) on the sinus venosus of the frog, *Rana pipiens*, in both summer and winter. The sinus was freed from the attached atrium and pinned ventral side down onto a cover-slip coated with a thin layer of cured Sylgard^R (Dow-Corning) resin. The posterior vena cava was split open in an anterior-posterior direction and the incision extended anteriorly into the dorsal wall of the sinus. The

cut sinus wall was folded back and secured with pins to expose the inside of the ventral sinus wall. For some experiments, both right and left vagosympathetic nerves were dissected with the sinus and sucked into a single suction electrode. The wall of the sinus is usually 10-50 μm thick. With differential interference contrast optics (Nomarski), one can see clearly the cardiac muscle fibres in the sinus and, in favourable preparations, the varicosities along the autonomic axons. The recording and ionophoretic pipettes were positioned under Nomarski optics at a magnification of 500 times with a water-immersion objective of 1·5 mm working distance (McMahan & Kuffler, 1971).

Solutions. The volume of the preparation chamber was less than 0·3 ml. The bathing fluid was continually exchanged at a rate of 0·5–5 ml./min. The preparation was dissected in normal frog Ringer which contained 111 mM-NaCl, 2 mM-KCl, 2 mM-$CaCl_2$, and 1 mM-Na-HEPES (Na-N-2-hydroxyethyl piperazine-N'-ethane sulphonate) buffer, pH 6·8. In order to abolish spontaneous activity of the sinus during intracellular recording, the preparation was placed in normal Ringer containing D600, 1 μg/ml. (α-isopropyl-α [(N-methyl-N-homoveratryl)-γ-aminopropyl]. 3,4,5 trimethoxyphenyl acetonitrile-HCl) for experiments requiring nerve stimulation or in Mn^{2+}-Ringer for most other experiments. Both D600 and Mn^{2+} block the slow inward current of the action potential (review by Fleckenstein, 1977). Mn^{2+}-Ringer contained 105 mM-NaCl, 2 mM-KCl, 1 mM-$CaCl_2$, 4 mM-$MnCl_2$ and 1mM-Na-HEPES pH 6·8. Low chloride Ringer contained 108 mM-Na isethionate (2-hydroxy ethane sulphonic acid), 2 mM-K methyl sulphate, 1 mM-$CaCl_2$, and 1 mM-Na-HEPES, pH 6·8. High K Ringer was made by substituting KCl for NaCl in Mn^{2+}-Ringer on a molar basis. Solutions of nucleosides and nucleotides were made fresh before each experiment and the concentrations measured spectrophotometrically at 260 nm. Adenyl (3'→5')$_9$-adenosine [(Ap)$_9$-A] was purchased from Boehringer-Manheim; 6-(2-hydroxy 5-nitrobenzyl) thioguanosine ([2-amino 6-(2-hydroxy 5-nitrobenzyl) thio]-9-β-D-ribofuranosyl purine) was purchased from Calbiochem; dipyridamole [Persantin^R, 2,6-bis (diethanolamino-4,8 diperidino-pyrimido (5-4-d) pyrimidine] was a gift from Boehringer-Ingleheim; D600 was from Knoll-AG; all other chemicals were from Sigma Chemical Co. The purity of the nucleosides, nucleotides, and the adenyl oligonucleotide [(Ap)$_9$-A] were verified by thin-layer chromatography on cellulose plates with isobutyric acid-conc. NH_4OH-water (66 : 1 : 33) as solvent and on PEI cellulose plates developed with 0·5 M-LiCl, as described by Randerath & Randerath (1967).

Drugs were added to the bathing medium by switching the reservoir feeding the preparation chamber. The volume of the tubing from the reservoir to the chamber was approximately 1 ml. At the usual perfusion rates of 1-2 ml./min between 30 and 60 sec elapsed after switching the reservoir before the new solution reached the bath. The chamber fluid attained an equilibrium with a new solution an additional 30-60 sec after the new solution began to enter the bath.

Electrical recording. Glass micro-electrodes were pulled on a horizontal puller from capillaries containing a glass fibre. They were filled by injecting 3 M-KCl into the shaft and had resistances of 70–120 MΩ. Conventional recording and ionophoretic techniques were used as described previously (Hartzell, Kuffler & Yoshikami, 1975; Hartzell, Kuffler, Stickgold & Yoshikami, 1977).

Ionophoretic pipettes were fabricated from fibre-filled capillaries and back-filled with 2 M-ACh-Cl, 1 M-adenosine hemisulphate, or 1 M-5'-AMP Na salt. ACh pipettes had resistances of about 200 MΩ, whereas lower resistance pipettes (100 MΩ) were required for application of adenine compounds. Ionophoretic pipettes were positioned under visual control with Nomarski optics, usually onto the impaled muscle fibre within 50 μm of the recording electrode. Ionophoretic pipettes could be seen to dimple the surface of the cell when they touched. Braking currents of 4-8 nA were sufficient to prevent leakage of ACh or adenine compounds from the pipette (Hartzell *et al.* 1975). The current passed through the ionophoretic pipette was monitored by measuring the current required to maintain the bath at virtual ground potential with a feed-back circuit utilizing an operational amplifier.

The vagus nerves were sucked into a suction electrode and stimulated by means of 0·5 msec duration rectangular pulses of 5-15 V.

RESULTS

Acetylcholine

ACh produced a dramatic hyperpolarization of the cardiac muscle fibres in the isolated, arrested frog sinus venosus, as shown in Fig. 1. In this, and most subsequent experiments, the spontaneous beat of the sinus was arrested by placing the preparation in Ringer solution containing either 4 mM-$MnCl_2$ or D600, 1 μg/ml. ACh was applied on to the sinus muscle fibres in several different ways.

In Fig. 1*A*, ACh was released from the vagus nerve terminals by stimulating the vagosympathetic nerve trunks via a suction electrode placed 5–10 mm distal to the sinus. In Fig. 1*A* three superimposed traces are shown. Each response was produced by a train of one, two, or three stimuli delivered to the vagus nerve. The frequency of the pulses within each train was 20/sec. Stimulation of the nerve produces a hyperpolarization which begins with a latency due partly to conduction time in the nerve and partly to the mechanisms of the cholinergic response (see below). The peak amplitude of the hyperpolarization is graded and becomes larger with increasing number of stimuli. The time course, however, is largely independent of the number of stimuli in the train, although in these records the larger responses have slightly longer times-to-peak than the small responses.

Fig. 1*B* illustrates responses of the sinus muscle cells to ACh released from an ionophoretic pipette. The Nomarski optics used to visualize the preparation revealed the muscle fibres clearly so that the ionophoretic pipette could be positioned accurately on the surface of the impaled muscle fibre. In the example of Fig. 1*B* the ionophoretic pipette was positioned so that it dimpled the surface of a fibre within 50 μm of the recording electrode. The position of the ACh pipette was then adjusted to give the best response. Often it was necessary to penetrate a layer of connective tissue covering the myocytes to obtain the response with the highest sensitivity. This was done by passing current pulses through the pipette while slowly advancing it or by gently tapping the micromanipulator when the ACh pipette was touching the muscle surface. Once this layer was penetrated, minor adjustments of the ACh pipette position had little effect on the response. Muscle fibres were uniformly sensitive to ACh (manuscript in preparation). In Fig. 1*B*, three superimposed traces are shown. Each response was produced by a 5 msec duration pulse of ACh of different amplitude. The time courses of the responses produced by ionophoretic application of ACh were generally slower than the responses to nerve stimulation. Ionophoretically evoked responses have times-to-peak and half-decay times about twice those found for nerve-evoked responses. Both times-to-peak and half-decay times increase with the amplitude of the ionophoretic pulse. Preliminary experiments (unpublished) suggest that this represents a saturation process during responses to ionophoretic ACh. The response to ionophoretic ACh invariably began with a latency of 50 msec or more (Fig. 1*C*). (see also Purves, 1976; Hartzell *et al.* 1977; Hall-Smith & Purves, 1978).

ACh was also bath-applied by addition to the bathing fluid, as shown in Fig. 1*D*. The sinus fibre in Fig. 1*C* was spontaneously active and firing action potentials at a rate of about 12/min. 0·15 μM-ACh was added to the perfusion medium. After about 30 sec required to purge the perfusion lines of normal Ringer, the fibre

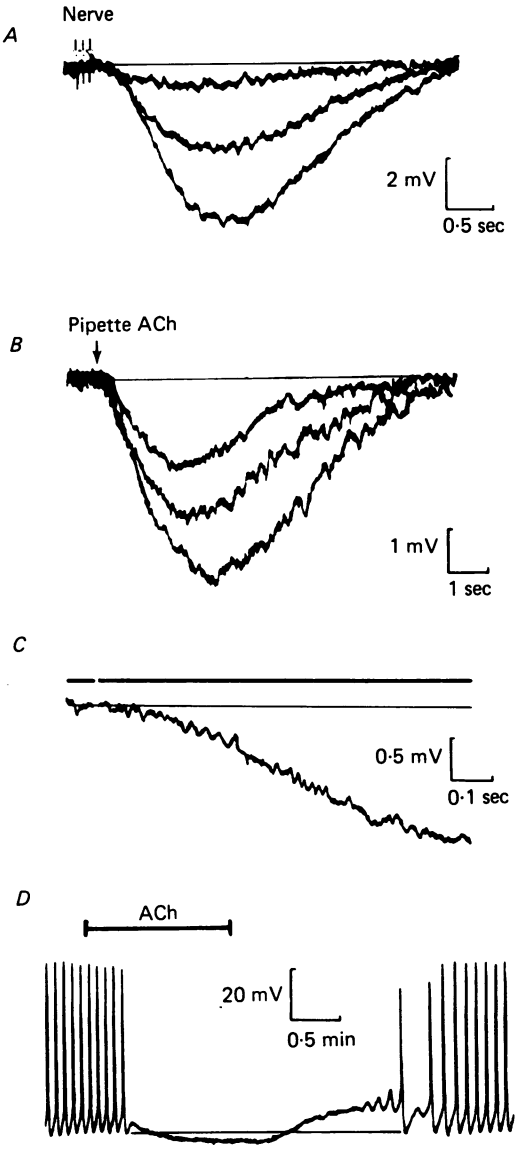


Fig. 1. Responses of sinus venosus muscle fibres to ACh released from various sources. *A*, three superimposed responses produced by stimulating vagus nerve with 1, 2, and 3 impulses. Preparation bathed in normal Ringer containing D600, 1 μg/ml. to suppress beating and 1 μM-propranolol to block sympathetic effects. *B*, three superimposed responses produced by 42, 65, and 135 nA, 5 msec duration ACh pulses. Preparation bathed in Ringer containing 4 mM-Mn^{2+}. *C*, delayed onset of response to ACh. A 5 msec, 50 nA pulse of ACh (upper trace) was applied to a sinus fibre to produce a 1·5 mV response (lower trace). The initial rising phase of the response begins about 50 msec after the voltage artifact produced by the ACh pulse. *D*, effects of bath-applied ACh on a spontaneously firing cell in the sinus venosus. 0·5 μM-ACh was added to the perfusion during the period indicated by the bar. Maximum diastolic potential is -58 mV. Records in *D* are from a chart recorder and action potential amplitude is attentuated.

hyperpolarized about 5 mV and spontaneous activity ceased. After removing ACh from the bathing medium, the cell repolarized and the membrane potential oscillated several times and triggered repetitive action potentials.

Few cells were found which were spontaneously active in the sinus bathed in 4 mM-Mn^{2+}-containing Ringer solution. The cells which were spontaneously active had overshooting action potentials which were completely abolished by TTX, 1 μg/ml. Occasionally, quiescent cells became spontaneously active for a period of several minutes during the recovery from bath-applied ACh or adenine compounds. Spontaneous activity could also be triggered by hyperpolarization of the membrane by passing current through a second intracellular electrode. Spontaneous activity was preceded by a period of oscillatory activity which occurred at a membrane potential between -50 and -60 mV.

Adenyl compounds

The response of the sinus venosus to adenine compounds is similar to, but slower than, the response produced by ACh. Fig. 2*A* shows the response of a sinus myocyte to pulses of adenosine delivered from an ionophoretic micropipette. In Fig. 2*A*, three traces are superimposed. Each response was produced by a 100 msec duration pulse of different amplitude. The largest pulse produces a response with a peak amplitude of about 7 mV which peaks in less than 3 sec after the ionophoretic pulse and takes 6 sec to decline to half of its peak amplitude. Thus, the response to ionophoretically applied adenosine may be somewhat slower than the response to ionophoretic ACh (compare Figs. 1*B* and 2*A*). In addition, the response to adenosine begins with a finite latency of about 300 msec after the beginning of the ionophoretic pulse (Fig. 2*B*). In Fig. 2*B* a response with a peak amplitude of 7 mV is shown on a fast timebase to emphasize the latency. For more than 200 msec after the onset of the ionophoretic pulse, there is no change in the membrane potential of the cell. It seems unlikely that the latency was due to diffusion of adenosine to distant receptors since the best responses were obtained when the ionophoretic pipette was touching the surface of the impaled muscle fibre. Similar results were obtained with ionophoretic application of 5′-AMP or ATP.

Fig. 2*C* illustrates the effect of bath-applied ATP on a spontaneously active fibre. 5 μM-ATP was bath applied to the preparation by addition to the perfusion fluid for the period indicated by the bar. The effect of 5 μM-ATP is remarkably similar to that produced by ACh in Fig. 1*C*. ATP hyperpolarizes the sinus fibre about 5 mV and inhibits the spontaneous activity. After washing out the ATP, the cell repolarizes and spontaneous activity is renewed.

Dose–response relationships for ACh and ATP

Fig. 3 compares dose–response curves for bath-applied ACh and ATP obtained from the same cell. Increasing concentrations of agonist were applied in a stepwise manner to a sinus fibre by addition to the bathing fluid. The maximum response is plotted as a function of the concentration of agonist. The dose–response curves for both ACh and ATP resemble rectangular hyperbolas expected from simple saturation kinetics. The concentration of ACh required to produce a half-maximal response was 3×10^{-7} M; that for ATP was 2×10^{-6}M. Thus, ACh is about 7 times more potent than ATP in producing a hyperpolarization in this cell. The maximum

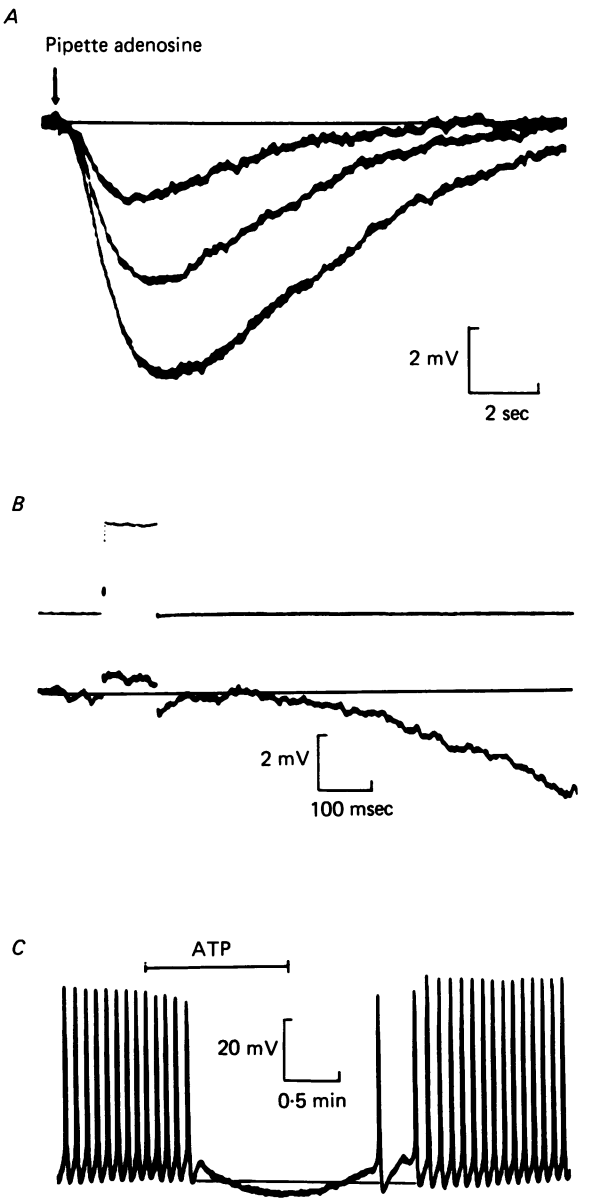


Fig. 2. Responses of sinus venosus muscle fibres to ionophoretic and bath-applied adenine compounds. *A*, three superimposed responses produced by 100 msec duration ionophoretic pulses of adenosine 75, 150, and 250 nA in amplitude. *B*, delayed onset of response to adenosine. A 100 msec, 250 nA pulse of adenosine (upper trace) was applied to the surface of a sinus muscle to produce a 7 mV response (lower trace). The initial rising phase of response begins about 300 msec after the start of the voltage artifact of the adenosine pulse. *C*, effects of bath-applied ATP on a spontaneously firing cell in the sinus. Same cell as illustrated in Fig. 1*C*. 5 μM-ATP (bar) produced a 5 mV hyperpolarization and cessation of spontaneous activity. Action potential amplitudes are attenuated by chart recorder.

response to ACh is always greater than the maximum response produced by ATP. In the cell of Fig. 3 the maximum response to ACh (produced by 10 μM-ACh is 34 mV, whereas the maximum response to 20 μM-ATP was 26 mV, or about 24 % less than the maximal ACh response. Dose–response curves for ATP and adenosine were identical.

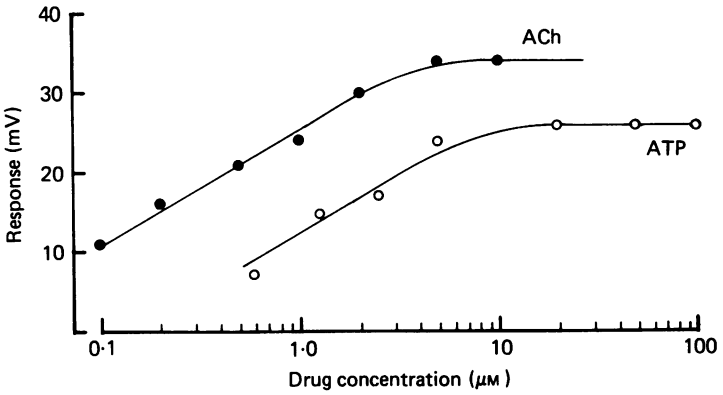


Fig. 3. Dose response curves for ATP (open circles) and ACh (filled circles). A sinus fibre was bathed in Mn^{2+}-Ringer and ATP or ACh added stepwise in increasing concentrations. The maximum hyperpolarizing response was recorded at a time after the membrane potential had stabilized (less than 1 mV change in 30 sec). All data from one cell. Resting potential was −58 mV.

The saturation with high doses of agonist is due partly to the membrane potential approaching the reversal potential of the response. Let us assume (*a*) that the responses to ACh and ATP are produced by identical ionic mechanisms (see below) involving only an increase in G_K and (*b*) that the potassium equilibrium potential (E_K) is −93 mV in 2 mM-KCl (Walker & Ladle, 1973, calculate E_K as −88 mV in 2·5 mM-external KCl). The observed values of the response can then be corrected for non-linear summation as described by Martin (1955) and Glitsch & Pott (1978). With this correction, the concentration of ACh required to produce a half-maximal response was $2{\cdot}7 \times 10^{-6}$ M and that for ATP was $4{\cdot}6 \times 10^{-6}$ M.

Antagonists of adenosine response

Several compounds block the response of the sinus myocytes to adenine compounds (Table 1). None of these compounds alone altered the membrane potential of the sinus cells more than several millivolts at the concentrations tested. The most potent antagonist was aminophylline.

Fig. 4 illustrates the effect of aminophylline on the response of the sinus venosus to bath-applied ATP and ACh. In Fig. 4*A*, 5 μM-ATP produces a hyperpolarization of 15 mV. In the presence of 10 μM-aminophylline, however, the response is reduced 50 %. The effect of aminophylline is specific to the responses produced by ATP: aminophylline has no effect on the response to ACh as shown in Fig. 4*B*. 0·15 μM-ACh produces a response of 10 mV both in the presence and absence of 10 μM-aminophylline.

Although quantitative comparisons were not made, theophylline had a similar effect to aminophylline. In the cell of Fig. 4, 50 μM-theophylline blocked the response to 5 μM-ATP about 70 % (not shown).

TABLE 1. Antagonistic effect of various agents on response of sinus venosus cells to ATP. 1 μM-ATP was bath applied onto a sinus fibre to produce a hyperpolarizing response. The concentration of antagonist (below) required to attenuate the hyperpolarization at least 50% was then measured by simultaneous bath application of 1 μM-ATP and the antagonist

Antagonist	Concentration (μM) to block hyperpolarization > 50%
Aminophylline	10
Papaverine	100
Isobutyl methyl xanthine	100
8-Br-ATP	200
8-Br-AMP	200
Quinidine	400

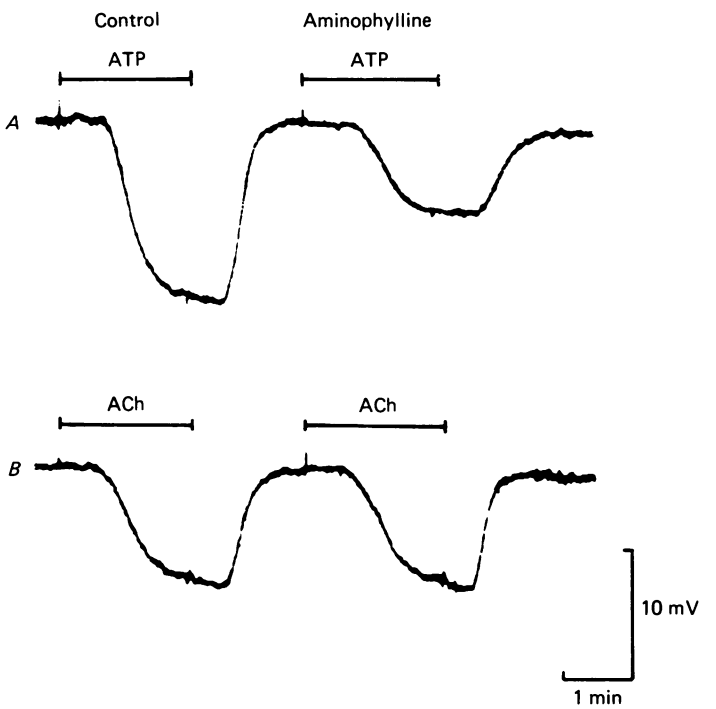
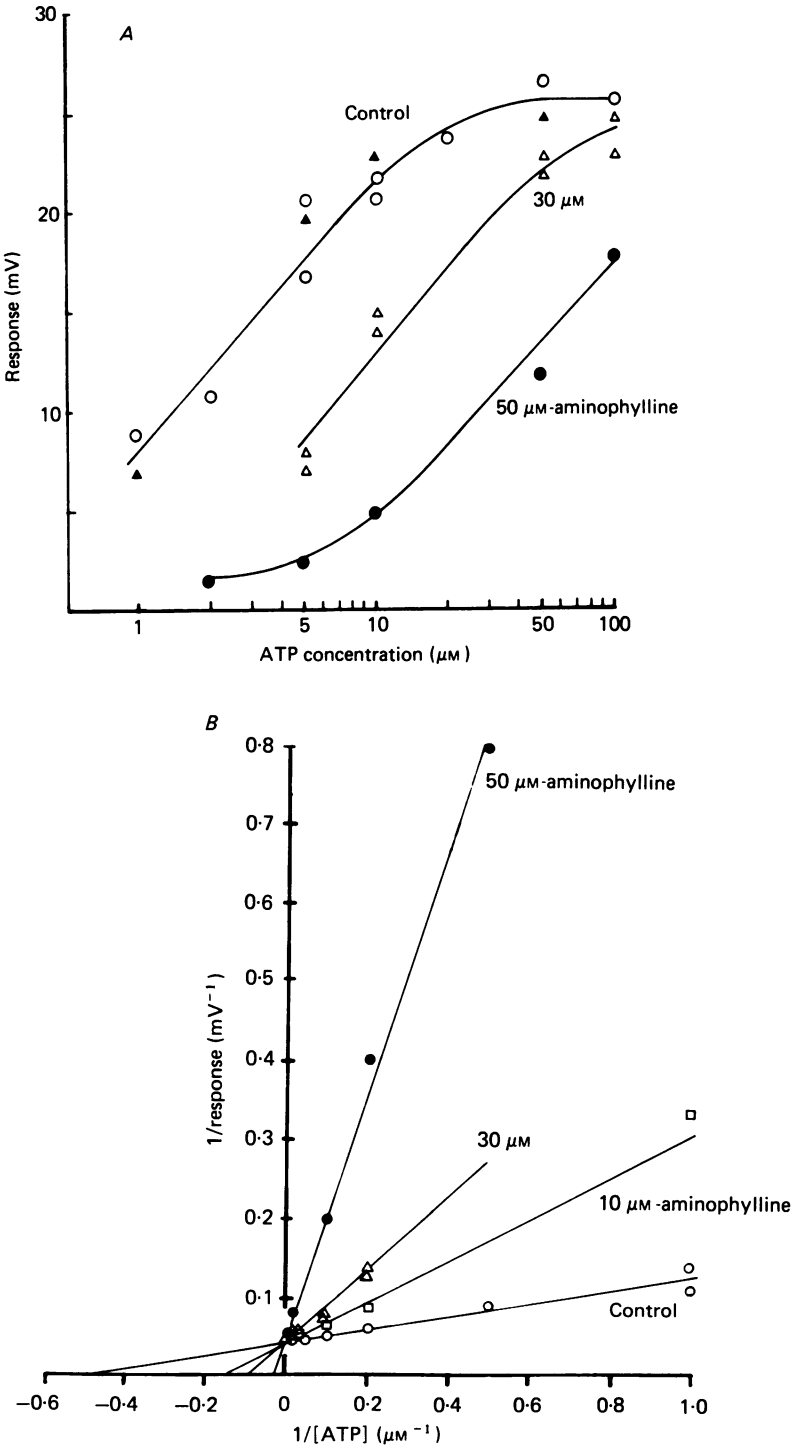


Fig. 4. Separation of cholinergic and ATP responses with aminophylline. *A*, responses produced in a sinus venosus fibre by bath application of 5 μM-ATP. *B*, responses in same fibre produced by 0·15 μM-ACh. Left traces: control responses. Right traces: responses produced by bath application of a mixture of ATP (*A*) or ACh (*B*) and 10 μM-aminophylline.

Aminophylline is a competitive antagonist. In order to test the nature of aminophylline inhibition, dose–response curves for ATP were determined in the presence of 0, 10, 30, and 50 μM-aminophylline (Fig. 5). Fig. 5*A* shows three dose–response curves, one obtained in the absence of aminophylline and the other two obtained in the presence of aminophylline (30 or 50 μM). The addition of aminophylline to


A
Control
30 μM
50 μM-aminophylline
Response (mV)
ATP concentration (μM)
B
50 μM-aminophylline
30 μM
10 μM-aminophylline
Control
1/response (mV⁻¹)
1/[ATP] (μM⁻¹)

the bathing solution shifts the dose–response curve for ATP to the right but does not affect the slope of the curve or the maximal ATP response.

These data, plus those obtained with 10 μM-aminophylline are plotted on a double reciprocal (Lineweaver-Burke) plot in Fig. 5*B*. in Fig. 5*B*, the lines obtained in

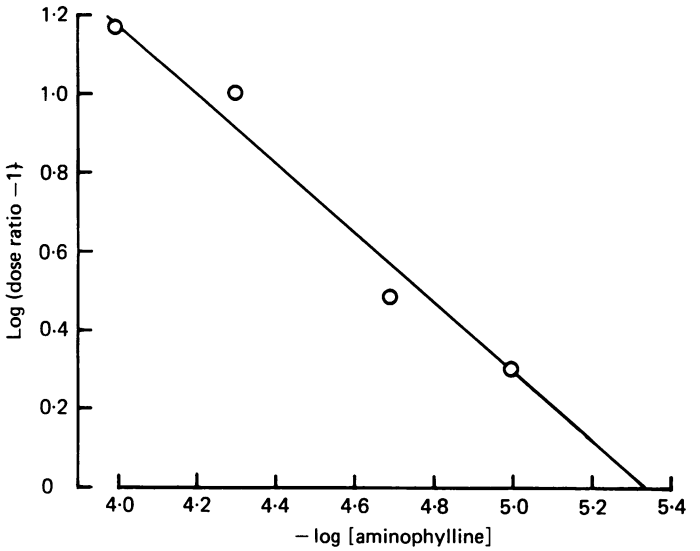


Fig. 6. Schild plot of aminophylline inhibition of adenosine response. Dose–response curves were determined for adenosine (hemi-sulphate) in the presence of 0, 10, 20, 50 and 100 μM-aminophylline. The ratio of the doses required to produce a 10 mV response in the presence and absence of aminophylline (the dose ratio) was determined. The line has a slope of −0·87 and extrapolates to the *X*-axis at 5·3.

different concentrations of aminophylline intersect the *Y*-axis at 0·04 mV^{-1} (equivalent to 25 mV response). These data suggest that aminophylline blocks the response to ATP by a mechanism of competitive inhibition. The apparent dissociation constant (K_{app}) for ATP binding was estimated to be 2·3 μM from the *X*-intercept (control curve, Fig. 5*B*). The K_i for aminophylline inhibition, calculated from the *X* intercepts of the curves obtained with aminophylline in the bathing medium (Fig. 5*B*) was between 4 and 5 μM.

Fig. 5. Effect of aminophylline on dose response curves to ATP. *A*, dose response curves plotted on semi-logarithmic co-ordinates. ATP was added to the bathing medium step-wise in increasing concentrations. The hyperpolarizing change of the membrane potential was recorded at a point when the membrane potential had stabilized (less than 1 mV change in 30 msec). Open circles (○): control dose response curve obtained in Mn^{2+}-Ringer. Open triangles (△): dose response curve after adding 30 μM-aminophylline to bathing medium. Filled circles (●): dose response curve after adding 50 μM-aminophylline. Filled triangles (▲): dose-response curve 20 min after removing 30 μM-aminophylline from bathing medium. The curve superimposes with the control curve (open circles). *B*, same data as in *A*, plotted on double-reciprocal (Lineweaver-Burke) plot. Open circles (○), control. Squares (■), 10 μM-aminophylline (data not shown in *A*). Triangles (△), 30 μM-aminophylline. Filled circles (●), 50 μM-aminophylline. Lines were fitted to the points by eye without weighting.

The apparent inhibition constant for aminophylline antagonism was also estimated using the method of Arunlakshana & Schild (1959). Dose–response curves for adenosine (hemisulphate) were determined in the presence of 0, 10, 20, 50, and 100 μM-aminophylline. The dose ratio (the ratio of the doses of adenosine required to produce a 10 mV response in the presence and absence of aminophylline) minus 1 is plotted *vs.* the negative log of the aminophylline concentration (Fig. 6). The line has a slope of $-0{\cdot}87$, which is close to the slope of -1 expected for a competitive inhibitor. The calculated pA_2 value is 5·3 which corresponds to a K_1 of 5 μM.

The effect of aminophylline is reversible as illustrated in Fig. 5*A*. The responses obtained approximately 20 min after washing out 30 μM-aminophylline (filled triangles (▲)) superimpose with the control responses obtained before treatment with aminophylline. The time course of reversibility was not examined critically. In at least some experiments, however, the recovery seemed to take some minutes. In the experiment of Fig. 5, a dose–response curve taken 4 minutes after washing out 30 μM-aminophylline had recovered only about 80 % and completely recovered after 20 min.

Structure—activity relationships

Adenyl compounds. The next series of experiments investigated the functional groups of adenine compounds important for hyperpolarizing activity (Table 2). In the cell of Table 2, similar concentrations of various adenyl compounds were bath-applied onto a sinus preparation and the steady-state hyperpolarization produced was recorded. Adenine and ribose exhibited no activity at concentrations of 0·5 mM, which was 1000-fold higher than was required for ATP to produce a sizeable hyperpolarization in all cells tested (see examples of Fig. 3). 1·7 μM-adenosine, however, produced an 18·5 mV hyperpolarization. Addition of phosphate groups to the 5′, 3′, or 2′ positions on the ribose of adenosine had no effect on the activity of the molecule: ATP, the adenosine monophosphates, and adenosine were virtually equipotent. Thus, hyperpolarizing activity is determined by the adenine and ribose moieties but is not greatly influenced by phosphate groups.

A systematic study was not made of the determinants on the ribose or adenine moieties important for activity. Several analogues of adenosine, however, were tested (Table 2). The effects of these analogues emphasized the importance of both the ribose and adenine groups. Certain modifications of the ribose moiety had strong effects on activity. In particular, the 2′- and 3′-deoxy derivatives of adenosine were approximately 100-fold less potent than adenosine in producing a hyperpolarization in the sinus cells. Likewise, certain modifications of the adenine moiety also had dramatic effects on potency. As described below, inosine, which lacks the 6-amino group of adenosine, is completely inactive at concentrations greater than 1 mM. Substitution of the hydrogen atom on position 8 of the purine ring with a bromine abolished activity. In addition, the 8-Br derivatives of adenosine acted as weak antagonists of the response to adenosine (Table 1). On the other hand, substitution of the hydrogen on position 2 of the purine ring with chlorine had little effect on biological potency.

Although adenosine monophosphates were equipotent with adenosine, adenosine *cyclic* monophosphates had a low potency relative to adenosine (Table 2). Both

TABLE 2. Effect of various adenyl compounds on the membrane potential of the sinus venosus. All data is from one cell. The listed concentration of ligand was bath-applied and the steady-state hyperpolarization produced was measured. The potency is % of the response produced by adenosine, in millivolts of response per umole agonist concentration

Ligand	Concentration (μM)	Response (mV)	Potency (%)
Adenine	500	0	0
Ribose	1000	0	0
Adenosine	1·7	−18·5	100
5′-AMP	1·6	−17·5	100
3′-AMP	1·6	−17·5	100
2′-AMP	1·7	−18·0	97
5′-ATP	1·5	−16·5	101
2′-deoxy adenosine	1·9	0	
	19·0	−1·5	0·7
3′-deoxy adenosine	22·0	0	
	109·0	− 6·0	0·3
2-Cl-adenosine	2·0	−18	83
8-Br-AMP	1000	0	0
8-Br-ATP	100	+ 1	0
3′ : 5′ cyclic AMP	1000	−17	1·6
2′ : 3′ cyclic AMP	1000	−20	1·8

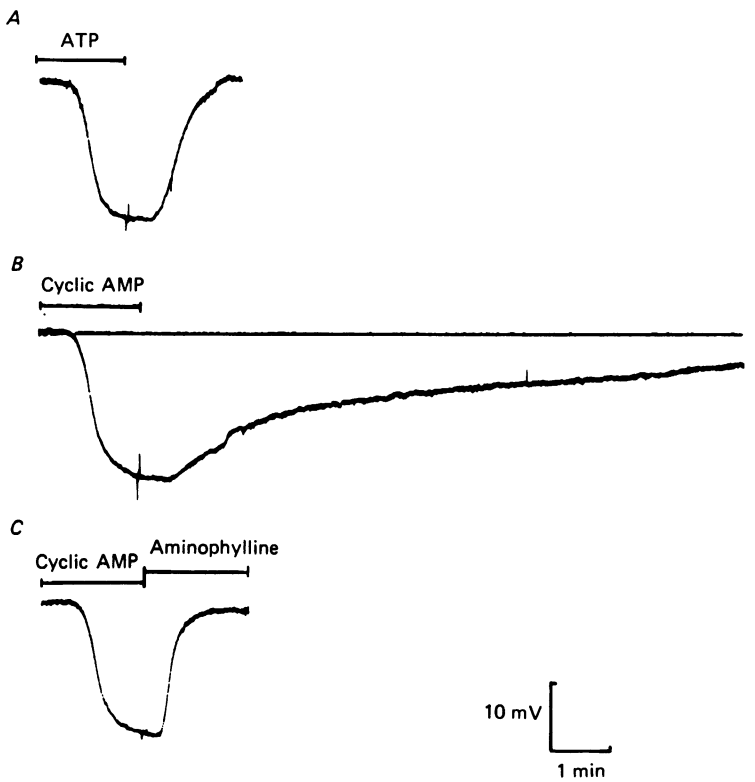


Fig. 7. Comparison of responses to ATP and cyclic AMP. *A*, response to 1 μM-ATP added to the perfusion fluid for the period indicated by the bar. *B*, response to 1 mM-adenosine 3′ : 5′ cyclic monophosphate (cyclic AMP). *C*, 1 mM-cyclic AMP was bath-applied as in *B*. The cyclic AMP was washed out with a solution containing 100 μM-aminophylline.

adenosine 3′:5′ cyclic monophosphate (3′:5′ cyclic AMP) and adenosine 2′:3′ cyclic monophosphate (2′:3′ cyclic AMP) were about 500-fold less potent than adenosine: concentrations of 1 mM were required to produce 20 mV hyperpolarizations. The time required for the membrane potential to return to resting levels after washing out the cyclic nucleotide, however, was very long (Fig. 7). In Fig. 7*A*, 1 μM-ATP produced a 20 μmV hyperpolarization which recovered about 1 min after washing out the ATP. 1 mM-3′:5′ cyclic AMP (Fig. 7*B*) produced a response with a steady-state amplitude similar to that produced by the 10^3-fold lower concentration of ATP, but the response lasted more than 10 min after washing out the cyclic nucleotide. Responses to ATP and cyclic nucleotide were blocked completely by 100 μM-aminophylline (not shown).

What are the mechanisms which produce this slow response to cyclic AMP? It was hypothesized that the cyclic nucleotides are removed from the muscle membrane more slowly than adenosine. This hypothesis was tested by observing the effect of aminophylline bath-applied during the washout of 3′:5′ cyclic AMP. It was reasoned that aminophylline would shorten the time course of recovery from 3′:5′ cyclic AMP if the long response were caused by repeated receptor activation by persisting agonist. In Fig. 7*C*, 1 mM-3′:5′ cyclic AMP was bath-applied to a sinus fibre to produce a 10 mV hyperpolarization. The cyclic AMP was removed from the bathing medium and replaced with control Ringer containing 100 μM-aminophylline. The time course of recovery of the membrane potential was greatly accelerated compared to that when 3′:5′ cyclic AMP was washed out with control Ringer (Fig. 7*B*).

Purine and pyrimidine nucleosides. Several different nucleosides were tested for their ability to hyperpolarize cells in the sinus venosus. The purine nucleosides, inosine and guanosine, and the pyrimidine nucleosides, cytidine and uridine, were completely inactive at concentrations of 1 mM. That is, none of these compounds at 1 mM altered the membrane potential more than 1mV in either direction. 5 μM-adenosine hyperpolarized the same cell more than 22 mV. Thus, adenosine is at least three orders of magnitude more potent than other common purine and pyrimidine nucleosides.

Do adenine compounds exert their effect pre- or post-synaptically?

The next experiment was designed to test whether adenine compounds produce their effects by acting directly on the cardiac muscle cells or by triggering release of ACh from nerve terminals which in turn would act on the cardiac cells. The involvement of ACh or other transmitters is excluded on several grounds. First, adenine compounds still produced hyperpolarizations in the presence of 4 mM-Mn^{2+} in the bathing medium (Fig. 2*A*). This concentration of Mn^{2+} completely blocks evoked transmitter release from the vagus nerve terminals (unpublished). Secondly, atropine completely blocks the response to ACh but has no effect on the response to ATP (Fig. 8). In Fig. 8, the responses of a sinus muscle fibre to bath-applied ACh and ATP were examined in the presence and absence of 50 nM-atropine. In control Ringer, 5 μM-ATP caused a 15 mV hyperpolarization (Fig. 8*A*) and 0·15 μM-ACh produced a 10 mV hyperpolarization (Fig. 8*C*). After addition of

50 nM-atropine, the response to ACh was completely abolished (Fig. 8*B*), whereas the amplitude of the response to ATP was unaffected (Fig. 8*D*). These data indicate that adenine compounds act directly on the cardiac muscle cell.

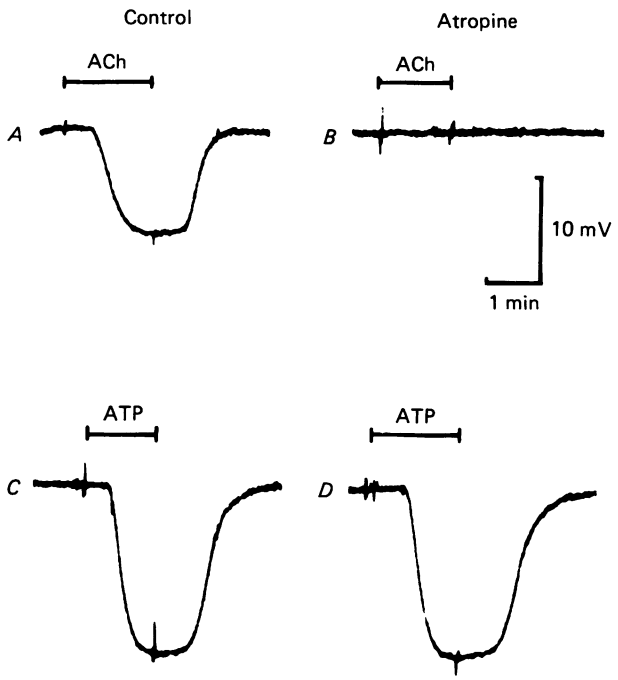


Fig. 8. Separation of cholinergic and adenosine responses with the muscarinic antagonist atropine. ACh or ATP was added to bathing medium for the period indicated by bar. *A*, response produced by 0·15 μM-ACh in control Mn^{2+}-Ringer. *B*, response to 0·15 μM-ACh is completely blocked after 50 nM-atropine is added to the bathing solution. *C*, response produced by 5 μM-ATP applied to same fibre. *D*, response to 5 μM-ATP is unaffected by addition of 50 nM-atropine to bathing medium. Cholinergic blocking action of atropine was slowly reversible.

Location of adenosine receptors

Are adenine compounds acting on receptors located on the surface of the muscle fibres or do they exert their effect intracellularly? Adenosine can enter cells by free diffusion and by mediated transport (active transport or facilitated diffusion). The experiments described below test the site of adenosine action by minimizing the permeation of adenosine into the sinus venosus cells.

Inhibition of active nucleoside transport. In order to reduce the permeation of adenosine into the sinus myocytes, adenosine transport was inhibited with potent blockers of the nucleoside transport system. The drugs that were used were dipyridamole (Plagemann & Roth, 1969; Kolassa, Pfleger & Rummel, 1970) and 6-(2-hydroxy 5-nitrobenzyl) thioguanosine (HNBTGR) (Paul, Chen & Paterson, 1975; Paterson, Naik & Cass, 1977). It was reasoned that blocking the transport system would have no effect or a potentiating effect on the hyperpolarizing action of adenosine if adenosine acts extracellularly, but would attenuate any effect of adenosine which required an intracellular site of action.

Fig. 9 illustrates the effect of dipyridamole on the response of the sinus cells to adenosine. In Fig. 9*A*, bath-applied 2 μM-adenosine produced a hyperpolarization of 12 mV. 20 μM-dipyridamole was then added to the bathing medium. Two minutes after addition of dipyridamole the hyperpolarization produced by 2 μM-adenosine had increased 50%, to 18 mV (Fig. 9*B*). Further, the time course of the recovery of the membrane potential to the resting level after washout of adenosine is greatly

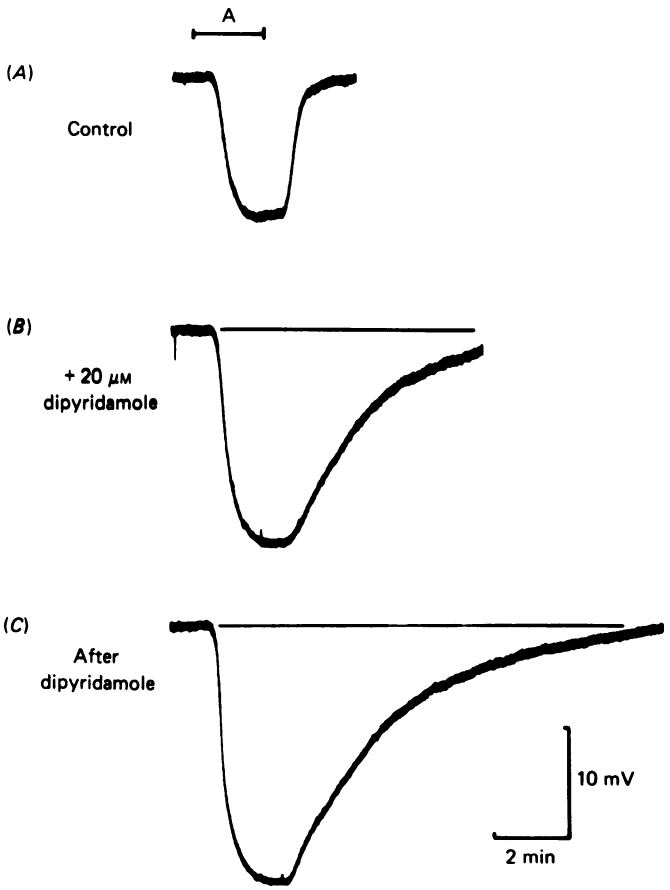


Fig. 9. Potentiation of response to adenosine with dipyridamole. *A*, control response of a sinus venosus cell to 2 μM-adenosine (*A*) added to the bathing medium for period shown by bar. *B*, response of the same cell 2 min after addition of 20 μM-dipyridamole to bathing solution. *C*, response of the same cell several minutes after removing dipyridamole from bathing medium. The response is further potentiated.

prolonged. In the control example (Fig. 9*A*), the membrane potential returns to resting levels 1·5 min after switching the perfusion back to control Ringer from adenosine-containing Ringer. When the bathing medium contains 20 μM-dipyridamole (Fig. 9*B*), the time of recovery is increased to about 8 min. The effect of dipyridamole is very slowly reversible. In the example of Fig. 9 the preparation was exposed to dipyridamole for a total of 12 min. After this time the dipyridamole was

washed out with control Ringer. The hyperpolarizing response produced by 2 μM-adenosine 10 min after washing out dipyridamole (Fig. 9*C*) is potentiated to 24 mV and lasts 10 min after removing adenosine from the bathing medium. The response to adenosine returned to control levels about 2·5 hr after removal of dipyridamole. Thus, dipyridamole clearly potentiates the action of adenosine.

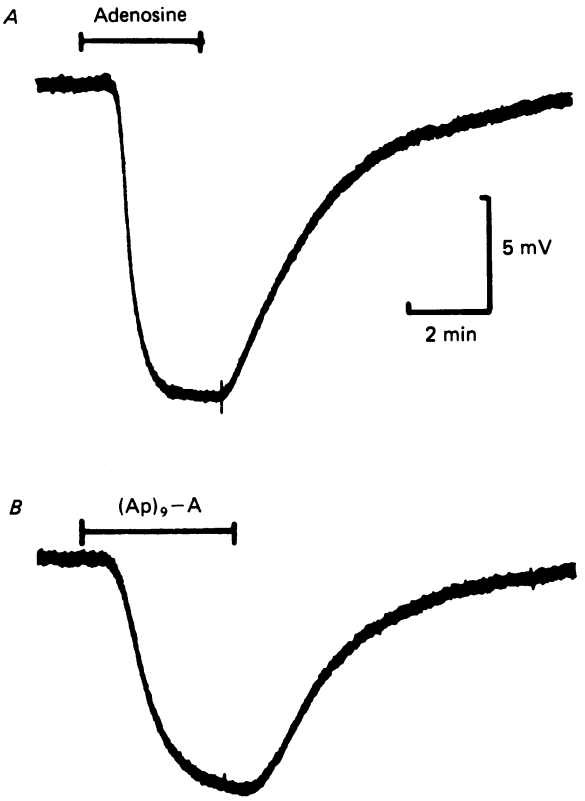


Fig. 10. Responses of a sinus venosus cell to adenosine and an impermeant adenyl oligonucleotide. *A*, response to bath-applied 2 μM- adenosine. *B*, response to bath-applied 0·4 μM-[adenyl (3′→5′)]$_9$-adenosine. This cell had been exposed briefly to 20 μM-dipyridamole an hour previous to this record.

Another drug which also inhibits adenosine transport, (2-hydroxy 5-nitrobenzyl) thioguanosine (HNBTGR), had similar effects to those of dipyridamole. For example, 1 μM-HNBTGR potentiated the steady state hyperpolarization produced by 2 μM-adenosine approximately 10%. In addition, the time course of recovery of the membrane potential after washing out adenosine was increased twofold.

Impermeant adenyl compounds. The second approach which was used to investigate the site of adenosine action was to test the response of sinus venosus cells to analogues of adenosine which permeate cell membranes very slowly. Two classes of molecules were used.

The first type of molecule that was used was a large molecular weight derivative

of adenosine which is too large to enter the cell intact. This molecule was the oligonucleotide, adenyl $(3'\rightarrow 5')_9$-adenosine, $[(Ap)_9\text{-A}]$. This oligonucleotide has a molecular weight of about 3300. The effect of this oligonucleotide on the membrane potential of a sinus cell is shown in Fig. 10. In Fig. 10*B*, 0·4 μM-$(Ap)_9$-A produces a hyperpolarization of 13 mV. In comparison (Fig. 10*A*) 2 μM-adenosine hyperpolarizes the cell 18 mV. Thus, on a molar basis, $(Ap)_9$-A is about 2·6-fold more potent than adenosine in producing a hyperpolarization in these cells. Since this oligonucleotide cannot enter the cell adenosine is most likely acting on the cell

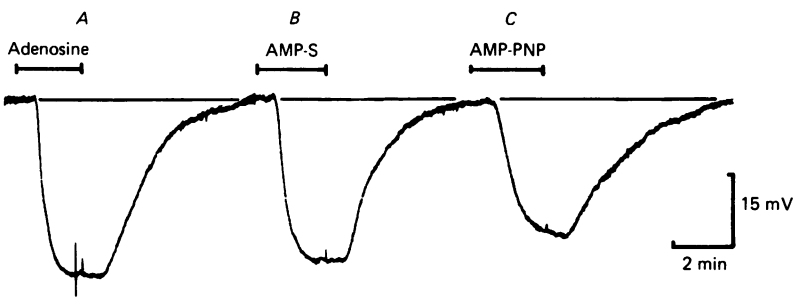


Fig. 11. Comparison of responses to adenosine and non-hydrolysable analogues of AMP. *A*, 2 μM-adenosine. *B*, 2 μM-adenosine 5'-0-thiomonophosphate (AMP-S). *C*, 2 μM-adenosine imidodiphosphate (AMP-PNP).

surface. To verify that my preparation of $(Ap)_9$-A was not contaminated with adenosine or mononucleotides, the $(Ap)_9$-A was subjected to thin-layer chromatography on polyethylenimine cellulose layers developed with 0·5 M-LiCl. The $(Ap)_9$-A was a single U.V.-absorbing spot with an R_f of 0. No U.V.-absorbing material was seen in the vicinity of 3'-AMP (R_f = 0·22), 5'-AMP (R_f = 0·22), or adenosine (R_f = 0·28).

The second class of molecules tested was adenine nucleotides unlikely to enter the cell. In general, nucleotides do no easily enter cells because of their negative charge. Since phosphohydrolase enzymes on cell surfaces can convert nucleotides to the nucleosides which are actively transported into the cell, several adenine nucleotides which are resistant to various phosphohydrolases were used. Adenosine 5'-0-thiomonophosphate (AMP-S) is an adenosine analog which is hydrolysed by 5'-nucleotidase at a rate less than 2% that of 5'-AMP (Murray & Atkinson, 1968). Adenyl imidodiphosphate (AMP-PNP) is an analogue which is not attacked by ATPases (Yount, Ojal, & Babcock, 1971; Melnick, deSousa, Maguire, & Packer, 1975). Fig. 11 illustrates the response of sinus myocytes to adenosine and the two analogues, AMP-S and AMP-PNP. In this example, 2 μM-adenosine and AMP-S both produce a hyperpolarization of 18 mV. AMP-PNP however, is about 25% less potent than adenosine or AMP-S: 2 μM-AMP-PNP produces a hyperpolarization of 14 mV.

Ionic mechanisms of adenosine response

It is generally accepted that ACh increases the permeability of the sinus membrane to K^+ (Noble, 1975) but what are the ionic mechanisms which mediate the response to adenosine and adenine nucleotides? The responses to ACh and adenine compounds have several similar features which suggest that they share similar ionic mechanisms. First, as shown in Fig. 3, the maximal hyperpolarizations produced by ACh and ATP are similar, although not identical. In the cell of Fig. 3, the maximum mem-

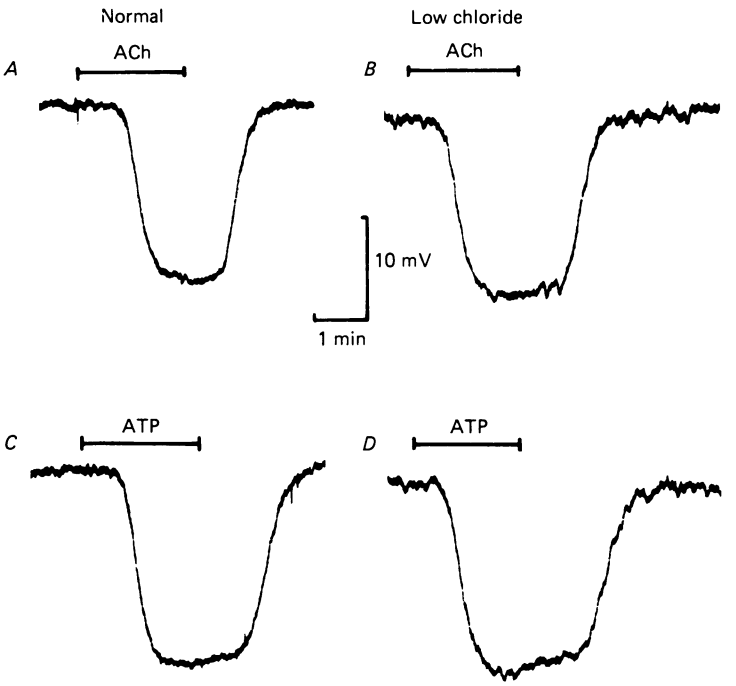


Fig. 12. Effect of low chloride Ringer on responses to ACh and ATP. *A*, response produced by bath-application of 0·15 μM-ACh in control (123 mM-Cl^-) Mn^{2+}-Ringer. *B*, response produced by 0·15 μM-ACh in low Cl^--Ringer. *C*, response produced by bath application of 2 μM-ATP in control (123 mM-Cl^-) Mn^{2+}-Ringer. *D*, response produced by 2 μM-ATP in low Cl^--Ringer. Low Ringer had all but 6 mM of the Cl^- ions replaced with impremeant isothionate ions (see Methods). Responses in low Cl^- were taken 3 min after switching the bathing solution to low Cl^-.

brane potential produced by ATP application was −84 mV; that for ACh was −92 mV. Since the maximum response tends towards the reversal potential for the response, the results of Fig. 3 suggest that the responses to ATP and ACh have reversal potentials more negative than −84 mV. The ions which may have equilibrium potentials in this range were K^+ and chloride.

Chloride. Fig. 12 shows the result of replacing most of the extracellular chloride (all but 6 mM) with impermeant isethionate. In Fig. 12*A*, 0·15 μM-ACh produced a 17·5 mV hyperpolarization in Ringer solution containing normal chloride (123 mM). When all but 6 mM of the chloride is replaced with impermeant isethionate ions, the membrane hyperpolarizes several millivolts, but the response to ACh is unaffected

(Fig. 12*B*). Likewise, the responses produced by 2 μM-ATP are identical in normal and low chloride Ringer (Fig. 12*C*, *D*).

Potassium. Increasing extracellular K^+ from 2 to 20 mM had similar effects on the responses to ACh and adenosine. In a typical experiment, the responses to ACh and ATP were both reduced 75–78% by increasing $[K^+]_o$ tenfold from 2 to 20 mM. However, since membrane resistance was not monitored in these experiments, one cannot rule out the possibility that the effect of increasing $[K^+]_o$ on responses to ACh and adenosine was due to a decrease in membrane resistance.

These results suggest that an increase in permeability of the membrane to K^+ or a decrease in permeability to Na^+ are at least partly involved in the response to adenine compounds but that changes in chloride permeability play little or no such role.

Is the response to ATP mediated by a Na-K pump?

The preceding experiments do not rule out the possibility that the response to ATP is mediated by a Na^+/K^+ pump. To test this possibility, the response of the sinus venosus to ATP was examined in the presence and absence of ouabain, an inhibitor of the Na^+/K^+ pump. In Fig. 13, 5 μM-ATP produced a 14 mV hyperpolarization. After addition of 20 μM-ouabain to the bathing fluid for 22 min, the cell depolarized about 2 mV. The response to ATP, however, was increased slightly, to 18 mV. After removing the ouabain, the membrane potential slowly repolarized 2 mV to the resting level.

Since ouabain has no effect, or perhaps a slight potentiating effect, on the response to ATP, it is concluded that a ouabain sensitive Na^+/K^+ pump is not involved in the response to ATP.

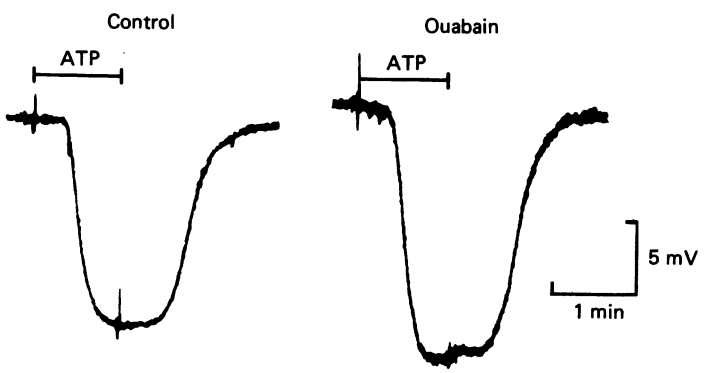


Fig. 13. Effect of ouabain on response to ATP. Left trace: response of a sinus venosus fibre to bath-applied 5 μM-ATP. Right trace: response of same cell to 5 μM-ATP 22 min after adding 20 μM-ouabain to the perfusion fluid. This concentration of ouabain produced a reversible depolarization of 2 mV and increased the noise of the record.

Does adenosine play a neurotransmitter role?

Since adenosine and adenine nucleotides are released from cholinergic nerves and have been shown to have a neurotransmitter role in other systems (see Discussion), several experiments were performed to determine whether adenine compounds

play a neurotransmitter role in the heart. Since it was shown in Fig. 8 that atropine completely blocks the response of sinus cells to ACh but has no effect on the response to ATP, I tested the effect of atropine on nerve-evoked responses. The results in Fig. 14 confirm the results of many other investigators; that the response of the heart to vagus nerve stimulation is blocked completely by atropine. In Fig. 14 *A*,

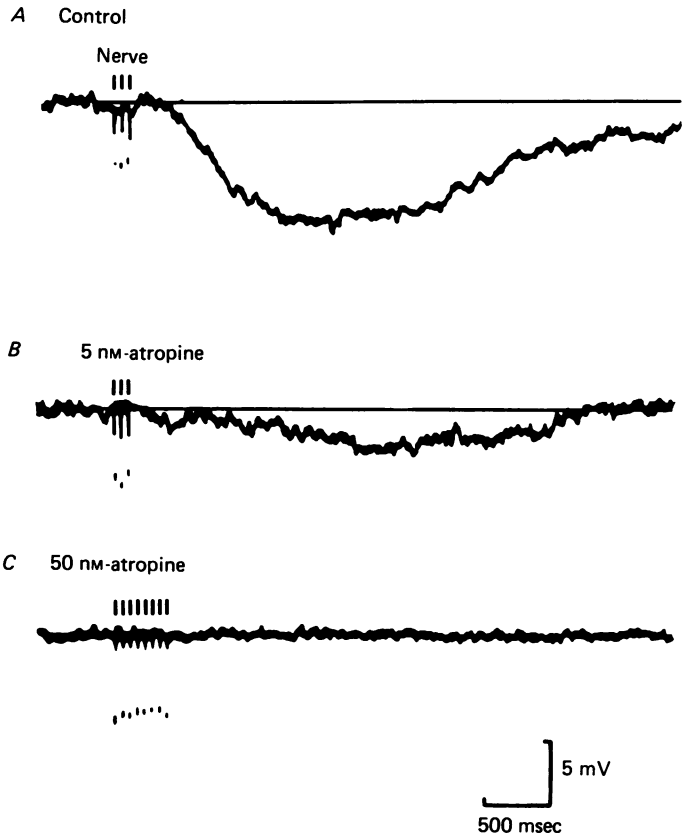


Fig. 14. Effect of atropine on response of sinus fibre to vagus nerve stimulation. *A*, control response produced by stimulating vagus nerve via a suction electrode with a train of three stimuli delivered at 20/sec. *B*, response of the same cell about 20 min, after adding 5×10^{-9}M-atropine to the bathing Ringer. *C*, after increasing the atropine concentration to 5×10^{-8}M the response is completely abolished even when a train of eight stimuli is delivered to the vagus nerve. This preparation was bathed in normal Ringer containing D600, 1 μg/ml.

three stimuli were applied to the vagus nerve and a 9 mV hyperpolarization was produced. Addition of 5 nM-atropine blocked the response about 75% (Fig. 14*B*). When the bathing concentration of atropine was increased to 50 nM, the response to vagus nerve stimulation was completely abolished (Fig. 14*C*). In Fig. 14*C*, eight stimuli were applied to the vagus nerve and no detectable hyperpolarization was recorded.

DISCUSSION

It has been proposed that adenosine and its phosphorylated derivatives have a strong effect on the sinus pacemaker region of the heart, since perfusion of the artery feeding the sinus node of the dog with solutions containing adenine nucleotides produces a dramatic sinus bradycardia (James, 1965). The current investigation, however, is the first which has examined directly the action of adenosine on the isolated sinus pace-maker. This study shows that adenosine and adenine nucleotides produce a long-lasting hyperpolarization of the membrane of sinus myocytes. This response, like that produced by ACh, is most likely mediated by an increase in the permeability of the membrane to K ions.

Adenosine receptors on the surface of sinus myocytes. Structure-activity relationships (Table 2) strongly suggest that the action of adenosine is a specific one and is mediated by interaction with a specific receptor molecule. The hyperpolarization produced by adenine compounds requires both adenine and ribose moieties, specifically the 6-amino group on the purine ring and 2′- and 3′-hydroxyl groups on the ribose. Purine and pyrimidine nucleosides other than adenosine are inactive. Since the hyperpolarization is blocked by low concentrations of aminophylline, this receptor resembles the P1 purinoreceptor described by Burnstock (1978).

Ionophoretic application of adenosine to the surface of sinus myocytes produces a hyperpolarization even when the preparation is bathed in a medium containing 4 mM-Mn^{2+} (Fig. 2*B*) which blocks secretion of neurotransmitters from the autonomic nerves. Thus, the receptor mediating membrane hyperpolarization is located on sinus myocytes and not on other cell types. Further, adenosine does not act indirectly by stimulating release of ACh since atropine has no effect on the response to adenosine (Fig. 8). This is consistent with the results of others that the chronotropic or inotropic action of adenine compounds is not affected by atropine (e.g. Drury & Szent-Györgyi, 1929; de Gubareff & Sleator, 1965; Johnson & McKinnon, 1956; James, 1965).

Adenosine is rapidly taken up by cardiac myocytes and other cells by a specific system of facilitated diffusion (Pfleger, Volkmer & Kolassa, 1969; Wiedmeier, Rubio & Berne, 1972; Hopkins, 1973; see other references in Paterson *et al.* 1977). Thus, it was important to determine whether the hyperpolarization produced by adenosine was triggered by direct interaction of adenosine with a *surface* membrane receptor or whether an intracellular site of action was required. From three independent experiments, I conclude that the adenosine receptor is located on the surface membrane of sinus myocytes. (i) Two drugs (dipyridamole and HNBTGR) which block the nucleoside transport system in a variety of cells (see references in Results) potentiate rather than block the hyperpolarization produced by adenosine (Fig. 9). These experiments extend and confirm previous studies showing the potentiating action of dipyridamole on the inotropic and chronotropic effects of adenine compounds (e.g. Stafford, 1966; Hopkins, 1973; Pfleger *et al.* 1969). (ii) An oligonucleotide consisting of ten adenylyl residues was several times more potent per mole than adenosine in eliciting a hyperpolarization (Fig. 10) even though this molecule is impermeant to the cell membrane because of its large size. Recently, two laboratories have used other high molecular weight derivatives of adenosine to demonstrate the presence of adenosine receptors on the surface of coronary

myocytes. Adenosine analogs which were bound covalently to an oligosaccharide (mol. wt. more than 1000) produced vasodilitation when infused into the coronary artery (Olsson, Davis, Khouri & Patterson, 1976). This adenyl-oligosaccharide had a molar potency about 40% that of uncomplexed adenosine. Likewise, 5'-AMP covalently coupled to carbonic anhydrase (mol. wt. = 30,000) produced vasodilatation but was less than 20% as potent as adenosine on a molar basis (Schrader, Nees & Gerlach, 1977). These authors also present evidence for a surface receptor for adenosine on the surface of atrial myocytes. (iii) Nucleotides are known to permeate cell membranes slowly (Krause, Wollenberger, Fedelesova & Ziegelhöffer, 1977; Robison, Butcher, Øye, Morgan & Sutherland, 1965). Nevertheless, naturally occurring nucleotides and synthetic nucleotides which were resistant to dephosphorylation were equal or nearly equal to adenosine in their effect on the sinus membrane potential (Fig. 11). From these three experiments, I conclude that adenosine acts on a *surface* receptor on the sinus myocytes. Further, since drugs which block the nucleoside transport system potentiate the action of adenosine, it seems likely that adenosine is inactivated by uptake.

Conductance changes produced by ACh and adenosine. It is generally agreed that ACh produces an increase in the permeability of the sinus membrane to K^+ (Garnier *et al.* 1978; reviewed in Noble, 1975). In addition, ACh also decreases the slow inward current which flows during the plateau phase of the action potential (Ikemoto & Goto, 1975; Giles & Noble, 1976; Ten Eick *et al.* 1976).

The hyperpolarization which is produced by adenine compounds is similar to that produced by ACh: responses to both ACh and adenine compounds tend toward a maximum membrane potential in the range of -84 to -92 mV (Fig. 3). The hyperpolarization produced by adenine compounds could be due at least partially to increases in permeability of the membrane to Cl^- or K^+, both of which have equilibrium potentials more negative than the resting potential. Since a twenty-fold change in external $[Cl^-]$ has no effect on the hyperpolarization produced by ACh or ATP (Fig. 13), it is clear that chloride plays little role in the responses. These experiments, however, do not exclude the possibility that the response to adenosine may be mediated in part by changes in permeability of the membrane to sodium.

The negative inotropic action of adenosine has been ascribed to its effect on the shape of the action potential. Adenosine decreases the plateau phase and duration of the action potential (Johnson & McKinnon, 1956; Hollander & Webb, 1957; de Gubareff & Sleater, 1965; Schrader, Rubio & Berne, 1975). This is similar to the effect that ACh produces on the action potential by reducing the slow inward current. This suggests that, like ACh, adenosine may reduce the slow inward current which flows during the action potential plateau.

Molecular mechanisms. At least three steps are involved in the response of the muscle to ACh or adenosine: diffusion of agonist to the receptor, binding to the receptor, and activation of the ionic conductance changes. The responses to brief pulses of ACh or adenosine delivered from a micropipette (Figs. 1, 2) are quite slow. These responses begin only after finite latencies and last at least several seconds. Diffusion calculations indicate that during the latent period (almost 300 msec for adenosine in Fig. 2*B*), virtually all of the agonist has diffused away. Thus, diffusion

and binding of either ACh or adenosine to the receptor must occur quickly relative to the time course of the response. This suggests that activation of conductance changes is rate-limiting. The molecular nature of this rate-limiting step is not known (Hartzell *et al.* 1977; Hill-Smith & Purves, 1978). In recent years, however, Greengard (1976) and others have advanced the hypothesis that slow synaptic potentials in the nervous system are mediated by mechanisms involving activation of adenylate or guanylate cyclase systems. For example, it has been suggested that the response of the heart to ACh may be mediated by the cyclic GMP system (George, Polson, O'Toole & Goldberg, 1970; see also references in Brooker, 1977) and the response to noradrenaline by the cyclic AMP system (reviewed by Tsien, 1977).

Adenosine has been shown to stimulate adenylate cyclase activity and produce accumulation of cyclic AMP in a wide variety of cell types (see representative references in Malbon, Hert & Fain, 1978) including ventricular myocardium (Huang & Drummond, 1976). The increase in cyclic AMP is potentiated by nitrobenzyl thioguanosine and dipyridamole and is blocked by theophylline. Similar results have been reported in other systems and it has been concluded that the stimulation of adenylate cyclase by adenosine is mediated by a cell surface receptor (e.g. Clark, Gross, Su & Perkins, 1974; Malbon *et al.* 1978). In several systems, however, adenosine apparently inhibits adenylate cyclase (Londos & Wolff, 1977; Calker, Müller & Hamprecht, 1978; Malbon *et al.* 1978).

Physiological role of adenosine receptors. The physiological role of adenosine receptors in the heart is unknown. Early in the course of these studies the hypothesis that adenosine was acting as a neurotransmitter seemed attractive. Adenine nucleotides have been proposed as neurotransmitters in both the peripheral (Burnstock, 1972) and central (Krnjevic, 1974) nervous systems. Further, adenine nucleotides are stored with neurotransmitters in synaptic vesicles of both cholinergic and adrenergic nerves and are released during nerve activity (review by Ribeiro, 1978). The experiments presented in Fig. 14, however, suggest that adenine compounds play little or no role as neurotransmitters under the conditions of this experiment. These experiments, however, do not exclude the possible existence of an intramural perinergic neurone in the heart which is excited by a muscarinic action of ACh.

In addition to its direct effects upon cardiac excitability and rhythmicity, adenosine is also a potent vasodilator and has been proposed to play an important role in regulating coronary blood flow in response to the oxygen demand of the myocardium (Berne, 1963). In support of this hypothesis it has been shown that adenosine is released at a very slow rate from the normoxic myocardium and that this rate increases four- to five-fold during hypoxia (Schrader, Haddy & Gerlach 1977) or transient coronary occlusion (Foley, Herlihy, Thompson, Rubio & Berne, 1978). It is postulated that the released adenosine interacts with adenosine receptors in the vascular smooth muscle (Olsson *et al.* 1976; Schrader, Nees & Gerlach, 1977) to produce vasodilitation. Thus, blood flow to the heart is increased during periods of oxygen need. Likewise, adenosine released from the myocardium may act upon receptors in the sinus pace-maker to slow the rate and reduce the strength of heart beat. This in turn would reduce the rate of oxygen consumption.

Regardless of the role of adenosine in the healthy heart, it seems likely that

adenosine and adenine nucleotides released from damaged cells during prolonged periods of anoxia or infarcation will have profound influences upon cardiac rhythm. The concentration of adenine nucleotides in the heart is about 5 μmole/g of tissue (Schrader & Gerlach, 1976). Thus, even a 1000-fold dilution of the contents of lysed cells would leave the adenine compounds at a concentration sufficient to arrest the sinus. One should consider, therefore, the possibility that release of adenine compounds from the site of an infarct might be responsible for some cases of sinus arrest or arrhythmias which sometimes occur during myocardial infarction (James, 1962).

Recently, surface receptors for adenosine and the effect of adenosine on cyclic AMP levels have been described in different cell types (see above). The physiological significance of adenosine receptors in various cell types is not known, but their widespread occurrence suggests that sensitivity to adenosine may be a common property of cells and may reflect a basic feature of the biochemical machinery of the cell surface (such as a regulatory site on adenylate cyclase). Furthermore, the possibility that adenosine plays a fundamental role in intercellular communication should be considered. It is well known that adenine compounds are common constituents of cells and that cells can both take up and release nucleosides (see references in Paterson, Kim, Bernard & Cass, 1975). The ability of cells to produce a specific chemical and release it into the extracellular fluid, the presence of a surface membrane receptor specific for the chemical, and the presence of an uptake mechanism which would function to inactivate the chemical and terminate its action are features well suited to a system of intercellular chemical communication.

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