Effect of Acetylcholine on the Norepinephrine-Induced Positive Chronotropy and Increase in Cyclic Nucleotides of Isolated Rabbit Sinoatrial Node

TAKASHI TANIGUCHI, MOTOHATSU FUJIWARA, JEUNG JA LEE, AND HIROYOSHI HIDAKA

SUMMARY Transmural stimulation of, or application of nicotine to, the isolated rabbit sinoatrial (SA) node resulted in initial negative and late positive chronotropy. Simultaneous application of acetylcholine and norepinephrine produced a similar biphasic chronotropic effect. These procedures produced an initial increase in cyclic guanosine 3':5'-monophosphate (cyclic GMP) and a delayed elevation in cyclic adenosine 3':5'-monophosphate (cyclic AMP). The initial and late effects on rate and nucleotide levels were inhibited by pretreatment with atropine and propranolol, respectively. Pretreatment with atropine shortened the time of maximum increase in cyclic AMP level and heart rate from 3 to 1 minute after the simultaneous application of acetylcholine and norepinephrine and enhanced the positive chronotropic effect. Physostigmine prolonged the duration of the increase in cyclic GMP and negative chronotropic effect after the simultaneous application. These results suggest that when acetylcholine and norepinephrine are present simultaneously in the SA node region, the former interacts predominantly with muscarinic receptors and stimulates the cyclic GMP system, which in effect delays the cyclic AMP elevation and reduces the positive chronotropic effect of norepinephrine. However, these effects of acetylcholine cannot be explained solely on the basis of changes in the cyclic GMP level, because sodium nitroprusside produced a marked elevation of the cyclic GMP levels without decreasing the heart rate and did not affect the norepinephrine-induced increase in pacemaker rate and cyclic AMP. Sodium nitroprusside may affect cyclic GMP pools other than those susceptible to acetylcholine. These cyclic GMP pools may not exert chronotropic effects.

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This work was supported by the Japan Heart Foundation Research Grant for 1977, and by a Grant from the Nippon Tobacco and Salt Public Corporation.
A preliminary report of this work was presented at The Third International Conference of Cyclic Nucleotides, New Orleans, USA, July, 1977.

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Original Manuscript received June 12, 1978; accepted for publication May 14, 1979.

THE sinoatrial (SA) node of the mammalian heart receives its nerve supply from both the parasympathetic (James and Spence, 1966) and sympathetic (Shindler et al., 1968; Taniguchi et al., 1977) divisions of the autonomic nervous system and, although these two nerves reciprocally regulate the heart rate, the parasympathetic nerve predominates (Volle and Koelle, 1975). Carrier and Bishop (1972) found that when both acetylcholine and norepinephrine were present, the former had a greater effect on heart rate.

Previously, we reported (Yamasaki et al., 1974; Taniguchi et al., 1977) that cyclic adenosine 3':5'-monophosphate (cyclic AMP) in the SA node was involved in the positive chronotropic effect of norepinephrine. However, George and associates (1970) showed that perfusion of the rat heart with acetylcholine resulted in an elevation of cyclic guanosine 3':5'-monophosphate (cyclic GMP) level which paralleled the negative inotropic and chronotropic effects. Watanabe and Besch (1975) suggested that, in the isolated guinea pig ventricle, cyclic GMP mediates the antiadrenergic effects of acetylcholine by specifically antagonizing the inotropic actions of cyclic AMP.

Recently, Diamond et al. (1977) showed that sodium nitroprusside markedly increased cat atrial cyclic GMP levels but did not decrease the twitch tension developed by the atrial strips. Brooker (1977) showed that low concentrations of carbachol decreased guinea pig atrial contractility without increasing myocardial cyclic GMP levels. These observations suggest that cyclic GMP may not be involved in mediating the negative inotropic action of acetylcholine on the heart.

The present study is an attempt to correlate the level of cyclic nucleotides and the predominant parasympathetic influence on heart rate with the...
combined effects of norepinephrine and acetylcholine, applied exogenously or released endogenously. We also attempted to define the role of cyclic GMP in the mediation of the chronotropic effects of acetylcholine by comparing the effects of this cholinergic transmitter with those of sodium nitroprusside on heart rate and effects of norepinephrine.

Methods

We studied albino rabbits of both sexes, weighing 1.8–2.3 kg. Under ether anesthesia, the rabbits were exsanguinated from the common carotid arteries. The heart was removed, placed in the nutrient solution (see below), and the SA node preparation was dissected free. The preparation has been previously characterized (Taniguchi et al., 1977) as nodal tissue by several criteria, including (1) a high density of adrenergic nerve fibers and norepinephrine, (2) a higher cyclic AMP content than other atrial tissue, and (3) a lack of contractility. The pacemaker rate then was recorded as described previously (Taniguchi et al., 1977).

Recording of Pacemaker Rate

Briefly, the SA node preparation was fixed horizontally on a bipolar platinum stimulating electrode, endocardial surface uppermost, between hooks at a resting tension of 100–300 mg in a bath of 60-ml capacity. The hook anchoring one end of the SA node was connected to the lever arm of a force displacement transducer (Nihonkoden Kogyo Co.). The bathing solution was gassed with a mixture of 95% O2 and 5% CO2 and was maintained at 38 ± 0.5°C. The pH of the solution was 7.4. Constituents of the solution were as follows (mm concentrations): Na+, 137.4; K+, 5.9; Mg2+, 1.2; Ca2+, 2.5; H2PO4−, 1.2; HCO3−, 15.5; Cl−, 134; dextrose, 11.5. Preparations were allowed to equilibrate for 60 minutes, during which time the bathing medium was replaced twice.

Transmembrane potentials were recorded from single cells of the SA node by glass microelectrodes filled with 3 M KCl. The membrane potential was monitored on an oscilloscope (VC-7, Nihonkoden Kogyo Co.) and recorded on film or an ink-writing polygraph (SEN-1101, Nihonkoden Kogyo Co.). Using a bipolar platinum electrode placed on the endocardial surface, we stimulated the neural elements in the SA node transmurally for 3 seconds by a train of rectangular pulses, 30 V in amplitude and 1 msec in duration, at a frequency of 20 Hz by an electronic stimulator (SEN-1101, Nihonkoden Kogyo Co.). The SA node rate was taken as the average value of 10 measurements of the cycle length between action potentials or recorded on a heart rate meter connected with a polygraph (Sanei Sokki Co.).

The experiments that followed were carried out in preparations exposed to bathing media for 60 minutes before transmural stimulation or addition of nicotine (10−4 M), acetylcholine (10−6 M), or ace-
TABLE 1  Regional Difference in Cyclic GMP in Rabbit Heart

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cyclic GMP (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA node</td>
<td>884 ± 94</td>
</tr>
<tr>
<td>Right atrium</td>
<td>297 ± 88</td>
</tr>
<tr>
<td>Left atrium</td>
<td>210 ± 48*</td>
</tr>
<tr>
<td>Right ventricle</td>
<td>92 ± 20*</td>
</tr>
<tr>
<td>Left ventricle</td>
<td>85 ± 31*</td>
</tr>
<tr>
<td>Ventricular septum</td>
<td>65 ± 10*</td>
</tr>
<tr>
<td>Papillary muscle</td>
<td>100 ± 28*</td>
</tr>
</tbody>
</table>

Sample was kept in the nutrient solution for 60 minutes at 30°C before freezing. Each value is the mean ± SE of five determinations. SA node region did not include the right atrium. * Significantly different from SA node, P < 0.06.

Effect of Transmural Stimulation on Cyclic Nucleotides and Pacemaker Rate

Under the experimental conditions used, unstimulated control sinus rate was 112 ± 9.7/min (mean ± SE, n = 10). Such SA nodal rates were not significantly changed during an observation period of 10-30 minutes. Transmural stimulation of the SA node produced a biphasic effect on the pacemaker rate, an initial negative chronotropic phase followed by an increase in rate. Such responses to transmural stimulation were abolished by pretreatment with tetrodotoxin (10^-7 M), indicating that the responses are nerve mediated. The negative chronotropy was abolished by atropine (10^-6 M) (Table 2).

TABLE 2  Effect of Transmural Stimulation on Cyclic Nucleotides and Pacemaker Rate of the SA Node

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Incubation time (sec)</th>
<th>Pacemaker rate (% of control)</th>
<th>Cyclic GMP pmol/mg protein</th>
<th>Cyclic AMP pmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>0.58 ± 0.07</td>
<td>14.9 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Stim</td>
<td>3</td>
<td>73 ± 3.5*</td>
<td>1.34 ± 0.26*</td>
<td>14.9 ± 1.5</td>
</tr>
<tr>
<td>TTX + Stim</td>
<td>100†</td>
<td>0.62 ± 0.11†</td>
<td>14.8 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Atr + Stim</td>
<td>137 ± 6.8*†</td>
<td>0.60 ± 0.02†</td>
<td>20.3 ± 1.8*†</td>
<td></td>
</tr>
<tr>
<td>Pro + Stim</td>
<td>78 ± 7.2*</td>
<td>1.09 ± 0.27*</td>
<td>15.6 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>Stim</td>
<td>10</td>
<td>113 ± 6.1*</td>
<td>0.94 ± 0.11</td>
<td>18.8 ± 1.8</td>
</tr>
<tr>
<td>TTX + Stim</td>
<td>100†</td>
<td>0.67 ± 0.13</td>
<td>14.6 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>Atr + Stim</td>
<td>148 ± 1.3*</td>
<td>0.69 ± 0.05</td>
<td>19.8 ± 1.2*</td>
<td></td>
</tr>
<tr>
<td>Pro + Stim</td>
<td>96 ± 1.7</td>
<td>0.77 ± 0.11</td>
<td>14.7 ± 1.7</td>
<td></td>
</tr>
</tbody>
</table>

Preparations were preincubated for 60 minutes at 30°C and exposed to 10^-8 M tetrodotoxin (TTX), 10^-7 M atropine (Atr), or 10^-6 M propranolol (Pro) for the last 10 minutes before the stimulation (Stim), then stimulated transmurally for 3 seconds by a train of rectangular pulses of 30 V in strength and 1 msec in duration at a frequency of 20 Hz. The SA nodes were incubated for 3 and 10 seconds after termination of stimulation before freezing. Nonstimulated control pacemaker rate is 112 ± 9.7 (mean ± SE) per minute. Each value is the mean ± SE of 10 determinations.

* Significantly different from control value (P < 0.05).
† Significantly different from values of Stim, 3 or 10 seconds (P < 0.05).

Results

Regional Differences in Cyclic GMP Contents in Rabbit Heart

The regional distribution of cyclic GMP in the heart is shown in Table 1. As described in Methods, all experiments were carried out with preparations exposed to bathing media for 60 minutes before stimulation or drug application. Thus, the regional comparison was made in the preparations kept in the nutrient solution for 60 minutes. The content of cyclic GMP per milligram of tissue protein of the SA node was higher than in the remaining parts of heart.
FIGURE 1  Effect of transmural stimulation on pacemaker rate and on cyclic AMP and cyclic GMP concentrations in the SA node preparation. Preparations were preincubated for 60 minutes at 30°C, then stimulated transmurally for 3 seconds by a train of rectangular pulses of 30 V in strength and 1 msec in duration at a frequency of 20 Hz. Control pacemaker rate is 112 ± 9.7/min. Each value is the mean ± SE of 9-10 determinations. In this and all figures, * = P < 0.05 compared to control. 0 in time refers to termination of stimulation.

cyclic GMP elevation and negative chronotropic effect were prevented (Fig. 2). The increases in content of cyclic GMP and cyclic AMP were prevented by tetrodotoxin (10⁻⁷ M). The cyclic GMP increase was prevented by atropine (10⁻⁶ M), and the cyclic AMP increase was prevented by propranolol (10⁻⁶ M) (Table 2). With the concentrations used, atropine and propranolol alone did not significantly affect either the pacemaker rate or the cyclic nucleotide content (Table 4). Tetrodotoxin (10⁻⁷ M) also did not affect these parameters: rate,
Effect of Nicotine on Cyclic Nucleotides and Pacemaker Rate

The application of nicotine \((10^{-4}\text{ M})\) to the SA node also had a biphasic effect on pacemaker rate; an initial negative chronotropic phase was followed by a positive phase. The decrease in rate was abolished by atropine \((10^{-6}\text{ M})\), and the increase was abolished by propranolol \((10^{-6}\text{ M})\). Unlike nerve stimulation, the effects produced by nicotine were not abolished by tetrodotoxin \((10^{-7}\text{ M})\). This indicates that nicotine acts directly on nerve terminals and not through nerve conduction. The time course of the effect of nicotine on the contents of cyclic nucleotides and pacemaker rate is shown in Figure 3. The content of cyclic GMP increased from 0.64 ± 0.09 to 1.33 ± 0.21 pmol/mg protein after a 40-second exposure to nicotine \((10^{-4}\text{ M})\), and the content of cyclic AMP increased from 16.8 ± 1.5 to 25.1 ± 2.7 pmol/mg protein after a 3-minute exposure. The pacemaker rate decreased for the first 40 seconds, then increased gradually over 5 minutes. The initial changes in cyclic GMP level and heart rate were abolished by atropine \((10^{-6}\text{ M})\), and the late changes in cyclic AMP level and heart rate were abolished by propranolol \((10^{-6}\text{ M})\) (Table 3).

Effect of Acetylcholine on Cyclic Nucleotides and Pacemaker Rate

The relationship between the concentration of acetylcholine and changes in the level of cyclic GMP is shown in Figure 4. Incubation with \(10^{-8}\) to \(10^{-5}\text{ M}\) acetylcholine for 20 seconds resulted in a concentration-related increase in the content of cyclic GMP in the SA node. The concentration of acetylcholine required for the half-maximal decrease in pacemaker rate was \(5 \times 10^{-7}\text{ M}\). The time course of the effect of acetylcholine \((10^{-6}\text{ M})\) on the contents of cyclic nucleotides in the SA node and pacemaker rate is shown in Figure 5. The content of cyclic GMP increased from 0.62 ± 0.12 to 2.62 ± 0.53 pmol/mg protein after a 20-second exposure to acetylcholine \((10^{-6}\text{ M})\) and reverted toward the control level 2 minutes later. The pacemaker rate decreased 20 seconds after application of acetylcholine, and this decrease lasted over 2 minutes. In contrast, there was no significant change in the level of cyclic AMP. These changes in the content of cyclic GMP and pacemaker rate were abolished by pretreatment with atropine \((10^{-6}\text{ M})\) (Table 4). On the other hand, pretreatment with physostigmine \((10^{-7}\text{ M})\) prolonged the acetylcholine-induced increase in cyclic GMP level and enhanced the negative chronotropic effect (Fig. 5). The effect of norepinephrine \((10^{-5}\text{ M})\) on cyclic nucleotides and pacemaker rate also is summarized in Table 4.

Effect of Simultaneous Application of Acetylcholine and Norepinephrine on Cyclic Nucleotides and Pacemaker Rate

The simultaneous application of acetylcholine and norepinephrine to the SA node produced a biphasic effect on the pacemaker rate, as in the case
of transmural stimulation or application of nicotine. The time course of the effect of simultaneous application of acetylcholine (10^{-6} M) and norepinephrine (10^{-5} M) on cyclic nucleotides and pacemaker rate is shown in Figure 6. There were initial increases in the cyclic GMP level and late elevations in the cyclic AMP level. The content of cyclic GMP increased rapidly over a 5-minute period. The initial increases in the cyclic AMP level or late elevations in the cyclic GMP level were attained 30 seconds later, whereas the content of cyclic AMP increased slowly from 17.5 ± 1.6 to 24.6 ± 2.0 pmol/mg protein, reaching maximum level after a 3-minute exposure. There were no initial increases in the cyclic AMP level or late elevations in the cyclic GMP level. The pacemaker rate decreased within the first 10 seconds, then increased slowly over a 5-minute period. The initial and late effects produced by simultaneous application of acetylcholine and norepinephrine were abolished by pretreatment with atropine (10^{-6} M) and propranolol (10^{-6} M), respectively (Table 5). Simultaneous treatment with both blocking agents completely abolished the initial and late effects of acetylcholine and norepinephrine.

As shown in our previous report (Taniguchi et al., 1977), the positive chronotropic effect of norepinephrine occurred immediately after application, in the absence of acetylcholine. The increase in cyclic AMP content after a single application of norepinephrine also occurred more rapidly than after a simultaneous application of acetylcholine and norepinephrine. These data are shown in Figure 6 and Table 6. Pretreatment with atropine (10^{-6} M) decreased the time required to attain a maximum increase in cyclic AMP from 3 to 1 minute after simultaneous application of acetylcholine and norepinephrine (Fig. 7). Such findings indicate that acetylcholine delays not only the onset of the positive chronotropic effect of norepinephrine but also the peak time of the norepinephrine-induced increase in cyclic AMP.

When simultaneous application of acetylcholine (10^{-6} M) and norepinephrine (10^{-5} M) was carried out in the presence of physostigmine (10^{-7} M), the maximum decrease in heart rate and elevation of the cyclic GMP level were attained 30 seconds later, and the cyclic GMP level remained increased for 2 minutes (Fig. 8). In the absence of physostigmine, the maximum effects were obtained at 10 or 20 seconds after application, and both heart rate and cyclic GMP level tended to return to the control level within 30 or 40 seconds (Fig. 6). Thus, physostigmine prolonged the duration of the negative chronotropic effect and of the increase in cyclic GMP levels.

**Table 3** Effect of Nicotine (10^{-4} M) on Cyclic Nucleotides and Pacemaker Rate of the SA Node

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Incubation time</th>
<th>Pacemaker rate (%) of control</th>
<th>cyclic GMP</th>
<th>cyclic AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>0.64 ± 0.09</td>
<td>16.8 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>Nic</td>
<td>40 sec</td>
<td>67 ± 8.5*</td>
<td>1.33 ± 0.21*</td>
<td>17.3 ± 2.0</td>
</tr>
<tr>
<td>Atr + Nic</td>
<td>132 ± 7.6†</td>
<td>0.62 ± 0.12†</td>
<td>25.9 ± 0.8†</td>
<td></td>
</tr>
<tr>
<td>Pro + Nic</td>
<td>68 ± 6.5*</td>
<td>1.80 ± 0.14*</td>
<td>18.9 ± 3.4</td>
<td></td>
</tr>
<tr>
<td>Nic</td>
<td>3 min</td>
<td>147 ± 10.5*</td>
<td>0.56 ± 0.18</td>
<td>25.1 ± 2.7*</td>
</tr>
<tr>
<td>Atr + Nic</td>
<td>137 ± 12.7*</td>
<td>0.66 ± 0.08</td>
<td>25.7 ± 1.2*</td>
<td></td>
</tr>
<tr>
<td>Pro + Nic</td>
<td>108 ± 13.7</td>
<td>0.61 ± 0.16</td>
<td>16.8 ± 1.2†</td>
<td></td>
</tr>
</tbody>
</table>

Preparations were preincubated for 60 minutes at 30°C and exposed to 10^{-6} M Atr or 10^{-5} M Pro for the last 10 minutes before addition of nicotine (Nic). Control pacemaker rate is 99 ± 8.3/min. Each value is the mean ± SE of six determinations.

* Significantly different from control value (P < 0.05).
† Significantly different from values of Nic at 40 sec or 3 min (P < 0.05).

**Discussion**

In our previous studies (Taniguchi et al., 1977), we found that the content of cyclic AMP was higher...
in the rabbit SA node than in the remaining parts of the atria. In the present work, the content of cyclic GMP in the SA node also was demonstrated to be higher than in the remainder of the heart. James and Spence (1966) showed that cholinesterase is present in large amounts in the SA node, atrioventricular node, and His bundle, in much smaller amounts in the right atrial myocardium, and not at all in the ventricular myocardium of the human heart. Such a high level of cyclic GMP in the SA node may be related to the density of cholinergic nerve terminals.

Our data suggest that the initial negative and late positive chronotropic responses to transmural stimulation of the SA node preparation are due to release of endogenous acetylcholine and norepinephrine. The responses mediated by the cholinergic transmitter, acetylcholine, appeared earlier than those induced by the adrenergic transmitter, norepinephrine. The earlier occurrence of the negative
The present results show that the content of cyclic GMP in the SA node is increased by an application of acetylcholine and that the cyclic GMP system in the SA node is linked to the muscarinic receptors. Such muscarinic receptor-linked changes in the cyclic GMP levels have been demonstrated in the heart (George et al., 1970; Watanabe and Besch, 1975; Gardner and Allen, 1976), smooth muscles (Schultz et al., 1973), secretory systems (Smith and Ignarro, 1975; Haymovits and Scheele, 1976), cultured neurons (Matsuzawa and Nirenberg, 1975), and brain slices (Ferrendelli et al., 1970; Hanley and Iversen, 1978). Our results are similar to such findings with respect to the transient accumulation of cyclic GMP in the SA node after an application of acetylcholine.

On the other hand, Brooker (1977) showed that cyclic GMP was not elevated by carbachol at a concentration equivalent to its ED50 for decreasing contractile force. In our study, we showed that cyclic GMP was elevated by acetylcholine at a concentration equivalent to its ED50 for decreasing the pacemaker rate. However, the elevation in cyclic GMP level did not precede the negative chronotropic effect at 10 seconds after application of acetylcholine (Fig. 5). There are three possible explanations for such a finding: (1) the cyclic GMP may be compartmentalized within the cell, as suggested by other investigators (Andersson, 1972), and changes in specific compartments, undetectable by total tissue measurements, may be important in the actions of acetylcholine; (2) negative chronotropic effects of acetylcholine may not be mediated by an increase in cyclic GMP content in the SA node; and (3) the effect of exogenously applied acetylcholine on cyclic GMP is different from that of acetylcholine released endogenously, since electrical stimulation produced simultaneous changes in heart rate and cyclic GMP, as seen in Figure 1.

The mechanism which acetylcholine delays the cyclic AMP elevation and reduces the positive chronotropic effect produced by norepinephrine in the SA node, and the reasons why acetylcholine responses appear earlier than those to norepinephrine recep-

### Table 4  Effect of Acetylcholine (10⁻⁶ M) or Norepinephrine (10⁻⁵ M) on Cyclic Nucleotides and Pacemaker Rate of the SA Node

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Incubation time</th>
<th>Pacemaker rate (% of control A)</th>
<th>cyclic GMP</th>
<th>cyclic AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control A</td>
<td></td>
<td>100</td>
<td>0.62 ± 0.12</td>
<td>16.8 ± 1.3</td>
</tr>
<tr>
<td>Atr</td>
<td>98 ± 0.8</td>
<td>0.58 ± 0.10</td>
<td>18.7 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>ACh</td>
<td>20 sec</td>
<td>62 ± 8.0*</td>
<td>2.62 ± 0.53</td>
<td>17.0 ± 2.0</td>
</tr>
<tr>
<td>Atr + ACh</td>
<td>99 ± 1.1†</td>
<td>0.66 ± 0.12†</td>
<td>18.0 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>Control B</td>
<td></td>
<td>105 ± 4.0</td>
<td>0.59 ± 0.10</td>
<td>19.0 ± 3.2</td>
</tr>
<tr>
<td>Pro</td>
<td>97 ± 3.0</td>
<td>0.54 ± 0.16</td>
<td>17.3 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td>3 sec</td>
<td>173 ± 16.0*</td>
<td>0.59 ± 0.04</td>
<td>30.0 ± 4.5*</td>
</tr>
<tr>
<td>Pro + NE</td>
<td>106 ± 3.0†</td>
<td>0.61 ± 0.09</td>
<td>17.3 ± 1.8†</td>
<td></td>
</tr>
</tbody>
</table>

Preparations were preincubated for 60 minutes at 30°C and exposed to 10⁻⁶ M Atr or 10⁻⁵ M Pro for the last 10 minutes before addition of 10⁻⁶ M acetylcholine (ACh) or 10⁻⁵ M norepinephrine (NE). Control pacemaker rate is 91 ± 0.3/min. Each value is the mean ± SE of six determinations.

* Significantly different from control value (P < 0.05).
† Significantly different from values of ACh or NE (P < 0.05).
The positive chronotropic effect of norepinephrine is associated with an increase in the content of cyclic AMP in the SA node. Murad et al. (1962) demonstrated that acetylcholine reduces the rate of cyclic AMP formation in a subcellular cardiac adenylate cyclase preparation; and Taniguchi et al. (1978) suggested that when intracellular levels of cyclic GMP increase, cyclic AMP hydrolytic activity of
the SA node phosphodiesterase is stimulated by cyclic GMP, and that this stimulation results in a reduction of the cyclic AMP level. Either a decrease in cyclic AMP synthesis or an increase in degradation would result in a reduction of cyclic AMP content which may, in turn, lead to suppression of the norepinephrine-induced chronotropic effect on the SA node.

In this study, we compared the effects of acetylcholine and sodium nitroprusside with respect to their ability to decrease the pacemaker rate and to antagonize the actions of norepinephrine on the pacemaker rate and cyclic AMP level. It was found that, although both agents produced a significant elevation of the cyclic GMP levels, only acetylcholine was effective in decreasing the pacemaker rate. Acetylcholine also delayed or reduced the effects of norepinephrine on the pacemaker rate and cyclic AMP levels, whereas sodium nitroprusside had no effect on the actions of norepinephrine. Thus, the negative chronotropic effect of acetylcholine and its antagonism to norepinephrine cannot be explained solely on the basis of changes in the cyclic GMP level. Additional or intermediate steps may be required for acetylcholine to exert these effects. Alternatively, cyclic GMP may not be the second messenger for the negative chronotropic effects of acetylcholine. It is also possible that acetylcholine and sodium nitroprusside may affect different pools of cyclic GMP and that the mechanism of increase in cyclic GMP levels differs with these two agents. Just as there is a dissociation of the effects of prostaglandins on cyclic AMP levels and heart rate (Süsskand et al., 1976), there may be pools of cyclic nucleotides not linked to the chronotropic response in the heart.

### Table 5
Effect of Simultaneous Application of Acetylcholine (10⁻⁶ M) and Norepinephrine (10⁻⁵ M) on Cyclic Nucleotides and Pacemaker Rate of the SA Node

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Incubation time (min)</th>
<th>Pacemaker rate (% of control)</th>
<th>cyclic GMP (pmol/mg protein)</th>
<th>cyclic AMP (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20 sec</td>
<td>84 ± 1.5*</td>
<td>0.58 ± 0.10</td>
<td>17.5 ± 1.6</td>
</tr>
<tr>
<td>ACh + NE</td>
<td>20 sec</td>
<td>110 ± 7.0†</td>
<td>2.13 ± 0.64*</td>
<td>16.0 ± 1.6</td>
</tr>
<tr>
<td>Atr + ACh + NE</td>
<td>3 min</td>
<td>85 ± 2.0*</td>
<td>1.63 ± 0.40*</td>
<td>16.5 ± 1.4</td>
</tr>
<tr>
<td>Pro + ACh + NE</td>
<td>3 min</td>
<td>97 ± 3.5†</td>
<td>0.56 ± 0.14†</td>
<td>17.0 ± 1.8</td>
</tr>
</tbody>
</table>

Preparations were preincubated for 60 minutes at 30°C and exposed to 10⁻⁶ M Atr and 10⁻⁵ M Pro for the last 10 minutes before simultaneous application of ACh and NE. Control pacemaker rate is 95 ± 2.5/min. Each value is the mean ± SE of six determinations.

* Significantly different from control value (P < 0.05).
† Significantly different from values of ACh + NE at 20 seconds or 3 minutes (P < 0.06).

### Table 6
Effect of Sodium Nitroprusside (10⁻⁵ M) on Norepinephrine (10⁻⁵ M)-Induced Increases in Cyclic Nucleotides and Pacemaker Rate of the SA Node

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Incubation time (min)</th>
<th>Pacemaker rate (% of control)</th>
<th>cyclic GMP (pmol/mg protein)</th>
<th>cyclic AMP (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20 sec</td>
<td>143 ± 1.7*</td>
<td>0.81 ± 0.11</td>
<td>24.6 ± 2.0*</td>
</tr>
<tr>
<td>ACh + NE</td>
<td>20 sec</td>
<td>146 ± 3.8*</td>
<td>0.53 ± 0.03</td>
<td>24.1 ± 1.1*</td>
</tr>
<tr>
<td>Atr + ACh + NE</td>
<td>3 min</td>
<td>143 ± 1.7*</td>
<td>0.81 ± 0.11</td>
<td>24.6 ± 2.0*</td>
</tr>
<tr>
<td>Pro + ACh + NE</td>
<td>3 min</td>
<td>146 ± 3.8*</td>
<td>0.53 ± 0.03</td>
<td>24.1 ± 1.1*</td>
</tr>
<tr>
<td>Atr + Pro + ACh + NE</td>
<td>3 min</td>
<td>143 ± 1.7*</td>
<td>0.81 ± 0.11</td>
<td>24.6 ± 2.0*</td>
</tr>
</tbody>
</table>

Preparations were preincubated for 60 minutes at 30°C and NE, sodium nitroprusside (Nitrop) or norepinephrine plus sodium nitroprusside (Nitrop + NE) were applied for 1 or 3 minutes. Control pacemaker rate is 104 ± 8.7/min. Each value is the mean ± SE of five determinations.

* Significantly different from control value (P < 0.05).
† Significantly different from values of NE per 1 or 3 min (P < 0.05).
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multiple comparison, and to M. Ohara of Kyoto University for preparing this manuscript.

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Acknowledgments

Thanks are due to Dr. A. Sakuma of Tokyo Medical and Dental University for his help in statistical analyses concerning

FIGURE 8  Effect of physostigmine on changes in pacemaker rate and cyclic AMP and cyclic GMP concentrations after simultaneous application of acetylcholine and norepinephrine to the SA node preparation. Preparations were preincubated for 60 minutes at 30°C and exposed to physostigmine ([T] = 1 M) for the last 10 minutes before simultaneous application of acetylcholine ([T] = 10−6 M) and norepinephrine ([T] = 10−5 M). □ = physostigmine non-treated pacemaker rate at preincubation time of 60 minutes; ■ = drug-treated pacemaker rate. Control pacemaker rate is 103 ± 7.6/min. Each value is the mean ± SE of six determinations.
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Circ Res. 1979;45:493-504
doi: 10.1161/01.RES.45.4.493

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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