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Correlation between cAMP Production in Guinea-Pig Left and Right Atria and Their Inotropic and Chronotropic Responses to Orciprenaline at Different Temperatures

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SUMMARY

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The positive inotropic responses to catecholamines are potentiated by cooling, whereas the positive chronotropic responses are inhibited. The possible site of this temperature-dependence was examined by determining the cAMP production in paced left and spontaneous right guinea-pig atria during orciprenaline-induced tension and rate responses. The time course of cAMP production preceded that of the respective responses at both 38 and 25°C. cAMP production in the left and right atria 15 seconds after addition of a concentration of orciprenaline (1.4×10^{-4} M) above that needed for maximum mechanical responses was determined at different temperatures in the presence of theophylline (10^{-3} M). These changes were compared with the effects of temperature upon complete tension and rate responses to a submaximal concentration of orciprenaline (9.5×10^{-8}). The increases in cAMP levels in the left and right atria and their respective mechanical responses were affected in parallel by temperature. The optimum temperatures for left (30°C) and right (38°C) atria were the same for cAMP and mechanical response. This close association is further evidence that cAMP plays an important role in these β-adrenoceptor mediated responses. Furthermore, the hypothermia-induced supersensitivity of the positive inotropic responses appears to be related to cAMP production.

INTRODUCTION

The hypothesis that the positive inotropic and chronotropic responses of the heart to β-adrenoceptor stimulation are mediated via stimulation of adenylate cyclase and the intermediate production of cAMP[2] has gained considerable support (1, 2). There is abundant evidence that the levels of this nucleotide are elevated before or during the responses to sympathomimetic amines. Additional evidence includes the prevention of both the cardiac responses and cAMP accumulation by β-adrenoceptor antagonists and the observation that the dibutyryl analogue of cAMP has cardiac stimulant properties (2). There is not, however, universal agreement (3, 4), controversy having arisen over several reports which dissociate the positive inotropic effects from the cAMP production (5-8).

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[2] The abbreviations used are: cAMP, cyclic 3',5'-adenosine monophosphate; COMT, catechol-O-methyl transferase; TCA, trichloroacetic acid.

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The present study is an alternative approach to the examination of this hypothesis. This is based upon the fact that the sensitivity of the heart to the positive inotropic (9, 10) and chronotropic (11–14) effects of β-adrenoceptor agonists is increased by lowering the temperature. The supersensitivity of the positive chronotropic responses can be entirely attributed to inhibition of COMT by the lowered temperature (12, 13, 15). When an agonist immune to COMT degradation such as orciprenaline (metaproterenol) is used, in fact subsensitivity can be demonstrated (15). In contrast, the positive inotropic responses to orciprenaline still exhibit a marked supersensitivity (15). The temperature optimum for the positive inotropic responses to single submaximal doses of sympathomimetic amines in guinea-pig atria is 25°C. This is clearly separated from the value of 37.5°C for the rate response (16, 17).

In view of the proposed link between the β-adrenoceptor and the adenylate cyclase enzyme system, the first objective of the present study was to examine the possibility that the temperature dependence is located at the level of cAMP production. The levels of cAMP were determined during orciprenaline-induced responses of guinea-pig isolated atria at different temperatures. In an attempt to relate this to the different effects of temperature upon the rate and tension responses, the cAMP levels in paced left atria (tension responses) and in spontaneously beating right atria (rate and tension responses) were determined simultaneously.

A further consequence of this study was to provide alternative evidence for the involvement of cAMP in mediating the positive inotropic and chronotropic responses to sympathomimetic amines. If the temperature affects the mechanical responses and cAMP production in a parallel manner, this might suggest that these effects are closely related.

METHODS

Guinea-pigs of either sex and weight range 300–500 g were killed by a blow on the head. The thorax was rapidly opened and the left and right atria removed separately as described previously (18). The atria were suspended in a 50 ml organ bath containing Krebs-bicarbonate solution (composition in mM: NaCl 118.4; KCl 4.7; $CaCl_2 2H_2O$ 1.9; $NaHCO_3$ 25; $MgSO_4 7H_2O$ 1.2; glucose 11.7; $NaH_2PO_4 2H_2O$ 1.2) gassed with 5% CO_2. Each atrium was connected to a transducer (Devices, Type UF1, 57 g sensitivity range) by a cotton thread, and their isometric tension recorded on a Devices M19 polygraph. An initial resting diastolic tension of 0.6–0.8 g was applied to both atria. The left atrium was paced at a constant rate of 2 Hz with square-wave pulses of 5 msec duration and a voltage of 50% above threshold, delivered by a SRI stimulator (Type 6053). Inotropic responses were obtained from this paced left atrium. Chronotropic responses were recorded by means of a ratemeter (Devices, Type 2751) triggered by the tension signal of the spontaneously beating right atrium.

All preparations were initially allowed to stabilize at a bath temperature of 38°C for 30 min, during which time several changes of the bathing medium were made. Maintenance of the temperature and cooling were achieved by means of a Churchill chiller circulator (CH/CTC/4).

cAMP determinations—tissue sampling. The bath temperature remained at 38°C or was lowered to either 30, 25 or 22.5°C, and after a 15 min equilibrium period a single dose (not exceeding 0.1 ml) of orciprenaline was added to the bath. At predetermined times after its addition the atria were rapidly removed from the bath, different sampling times being used for the individual atria of each pair. This was facilitated by having them mounted separately the left atria being tied to a pair of unshielded platinum electrodes and the right atria to a separate tissue holder. Both had rapid-release clips. Each atrium, attached to its holder, was immediately immersed in liquid nitrogen. Whole frozen atria were cut from their mounting, weighed and dropped into an ice-cold homogenizer consisting of a piston-type Teflon pestle and a glass grinding vessel of 2 ml working capacity, and containing 0.5 ml TCA (6% w/v). The homogenate was transferred to a centrifuge tube, to which was added a further 0.2 ml of TCA used for washing the homogenizer. After centrifu-

gation of the homogenate at 2,000 × *g* for 10 min, the supernatant was pipetted off and washed four times each with six volumes of water-saturated diethyl ether. Residual ether was removed by evaporation in a stream of air. One hundred milliliter aliquots of the washed extract were dried *in vacuo* over phosphorus pentoxide. The dried residues were taken up in 1 ml of 50 mM tris-HCl buffer (pH 7.4) containing 8 mM theophylline and 6 mM 2-mercaptoethanol; 50 μl aliquots of this solution were assayed.

Assay procedure. cAMP was measured by the radioisotope dilution assay method of Brown *et al.* (19). The binding protein was prepared from bovine adrenal glands which were collected as soon as possible after slaughter and transported on ice to a cold room at 4°C. The cortices were removed and each 2 g of this tissue were homogenized with 3 volumes of an ice cold medium, comprised of sucrose 0.25 mM; potassium chloride 25 mM; magnesium chloride 5 mM and tris-HCl buffer 50 mM. The supernatant obtained after centrifugation at 2,000 × *g* for 5 min was respun at 5,000 × *g* for 15 min. The resultant supernatant was stored at −20°C in 0.5 ml aliquots. A fresh aliquot was thawed and diluted with an appropriate volume of buffer for each assay. The volume of buffer was determined from a binding protein dilution curve, and was that dilution which bound 25% of tracer cAMP (20).

The assay tubes were prepared containing 50 μl of either a known amount (0–2.5 ng) of cAMP standard or the unknown sample, [8-^{3}H]cAMP (50 μl of 330 nCi/ml) and buffer (100 μl). These solutions were thoroughly mixed on a rotary mixer before addition of 100 μl of the binding protein. This procedure was repeated in triplicate for each concentration of the standard cAMP and for each sample. Control tubes, also in triplicate, were set up by replacing the binding protein with buffer (100 μl). The final solutions were thoroughly mixed and incubated at 0°C for a minimum of 90 min.

After incubation, 1 ml of ice-cold buffer was added to each tube and the solution filtered through buffer-moistened Millipore filters (25 mm, 0.45 μ). A further 1 ml of buffer was used to wash the filter and incubation tube. Each filter was placed in a scintillation vial and dried by incubating at 50°C for 30 min. The dry filters were dissolved in 7 ml of scintillation solution (1 part 2-(methoxy)ethanol to 4 parts of 0.8% w/v solution of 2,5-diphenyloxazol in toluene) by vigourous shaking. They were then counted for 10 min in a Beckmann scintillation counter (LS 235).

A calibration curve was obtained for each assay by plotting the concentration (pg) of the standard cAMP solutions against Co/Cx, where Co = (counts/min of the zero standard − control) and Cx = (counts/min of standard (sample) − control). Amounts of cAMP in unknown samples were determined by reference to the calibration curve. Because comparisons were made between left and right atria in which the procedures were identical, no correction was made for extraction recovery.

Effect of temperature upon complete submaximal responses to orciprenaline. The left and right atria were removed separately and set up as above except they were mounted on a combined perspex tissue holder (right atria) and electrode for pacing the left atria. After the initial equilibrium period at 38°C, the bath temperature was raised to 42.5°C and a single submaximal dose of orciprenaline added to the bath. This was left in contact with the tissue until the maximum effect was produced, when the bath was washed out to restore the resting rate and tension to their pre-drug level. This procedure was repeated at lower temperatures, a 15 min equilibrium being allowed before addition of the orciprenaline at each temperature.

Materials. Adenosine 3′5′ cyclic monophosphate (Sigma), [8-^{3}H]adenosine 3′5′ cyclic monophosphate (ammonium salt; 27 Ci/mmole) (The Radiochemical Centre, Amersham), (±)-orciprenaline sulphate (Boehringer Ingelheim) and theophylline (Sigma). Orciprenaline was freshly prepared in 0.9% w/v NaCl solution.

RESULTS

Determination of an appropriate concentration of orciprenaline. Using an arbitrary sampling time of 5 seconds after addition of the orciprenaline, a preliminary

concentration (4.7×10^{-7} M) that produced submaximal rate and tension responses, increased the mean cAMP level of the left atria from 181.0 ± 18.3 pg/mg (n = 5) to 222.0 ± 68.3 pg/mg (n = 3) at 38°C. This was not a significant ($p > 0.05$) increase and individual values showed a lack of consistency. However, a concentration (1.4×10^{-4} M) in excess of that required for maximal mechanical responses, produced consistently larger increases in cAMP above the resting level and was therefore used for all subsequent studies.

Time-course of cAMP production. The time courses for cAMP production by this concentration in the left and right atria were compared with the time courses for tension and rate responses, respectively, of the same tissues. The responses were measured as the increase in tension and rate above the pre-orciprenaline resting levels. Time courses at both 38 and 25°C were determined. The elevation of cAMP levels in the left atria preceded the changes in tension at both 38 and 25°C (Fig. 1). The peak increase in cAMP occurred 15 seconds after addition of the orciprenaline to the bath at both temperatures. However the rate of onset of the tension response was considerably slower at 25°C. The increase in cAMP levels of the right atria also preceded the rate responses of these preparations (Fig. 2). The peak increase in cAMP levels of the right atria, however, contrasted with that for the left, in that it occurred at 30 seconds at both temperatures (Fig. 2). The mean (n = 3) increase in cAMP at the peak in the right atria (332.4 ± 18.4 pg/mg) was also greater than that at the peak for the left atria (223.7 ± 58.9 pg/mg) at 38°C. At 25°C, however, the cAMP increases at the peak did not differ between left and right atria. Since the time for optimum production of cAMP did not vary between 38 and 25°C, a constant sampling time of 15 seconds was employed for all temperatures used and for consistency between the left and right atria.

Effect of the phosphodiesterase inhibitor theophylline. It was possible that the cAMP levels determined during the orciprenaline-induced responses were reduced by partial hydrolysis to 5'-AMP by phosphodiesterase. Furthermore, this enzyme

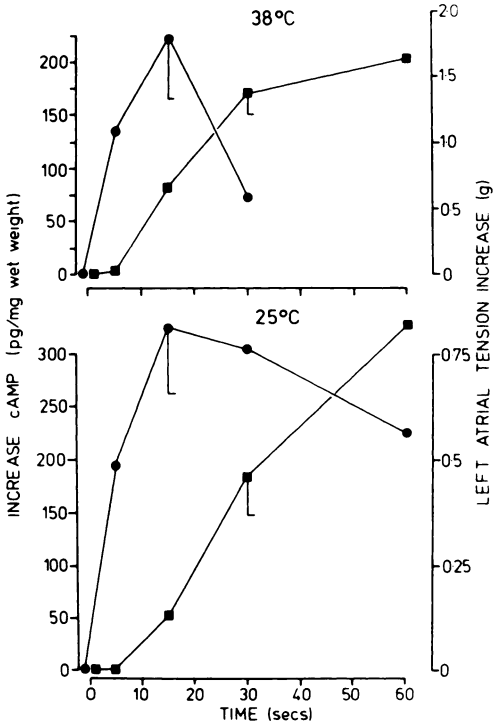

FIG. 1. *Time course of the increase in cAMP* (●) *and tension* (■) *of guinea-pig isolated left atria in response to orciprenaline*

At predetermined times after addition of orciprenaline (1.4×10^{-4} M) to the bath, the atria were rapidly removed to liquid nitrogen for determination of cAMP levels as described in METHODS. The mean (n = 3) increase was determined by subtracting the mean (n = 5) levels obtained without the orciprenaline (181.6 ± 18.3 pg/mg at 38 and 164.3 ± 20.7 pg/mg at 25°C). The increase tension at the time of removal was recorded. The bath temperatures were 38 (upper panel) and 25°C (lower panel). Vertical bars represent the SEM of that point on the curve.

may have been inhibited at the lower temperature. To avoid any possible interference by phosphodiesterase, the determination of cAMP levels at different temperatures was undertaken in the presence of the phosphodiesterase inhibitor theophylline (21). The levels of cAMP 15 seconds after the addition of orciprenaline were elevated by theophylline (10^{-3} M) (Fig. 3). This potentiation was not significant in the left atria, but was significant ($p < 0.05$) at both 38 and 25°C in the right atria.

Effect of temperature upon cAMP production. The mean (n = at least 4) orciprenaline-induced increases in cAMP in the

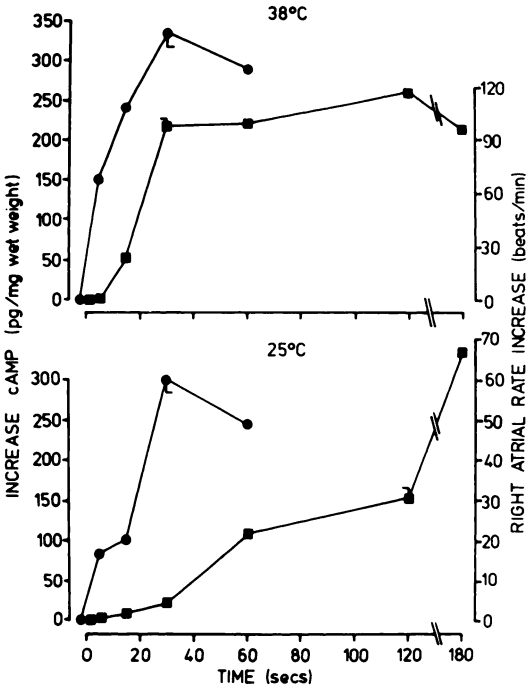

FIG. 2. *Time course of the increase cAMP (●) and rate (■) of guinea-pig isolated right atria in response to orciprenaline*

At predetermined times after addition of orciprenaline (1.4×10^{-4} M) to the bath, the mean (n = 3) increase in cAMP levels and increase rate were determined as described in Fig. 1. The bath temperatures were 38 (upper panel) and 25°C (lower panel). Mean (n = 5) basal levels of cAMP were 106.9 ± 11.2 pg/mg at 38 and 116.7 ± 17.8 pg/mg at 25°C. Vertical bars represent the SEM of that point on the curve.

left and right atria determined at 15 seconds into the response at 38, 30, 25 and 22.5°C are shown in Fig. 4. On cooling, the left atrial cAMP production increased to a plateau between 30 and 25°C, whereas in the right atria there was a progressive fall in cAMP production which was small at first but more marked at 25 and 22.5°C.

Effect of temperature upon rate and tension responses. Although both the physiological responses and the cAMP production of each tissue were determined, it was not possible to make a direct comparison between them since at the 15 second sampling time the rate and tension responses had barely commenced. This is evident from the time course experiments (Figs. 1 and 2). Separate experiments were therefore performed in which the responses were allowed to develop completely rather than terminating them at a set time as in the cAMP determinations. In addition, the concentration of orciprenaline used for the cAMP determinations was reduced to a submaximal level (9.5×10^{-8} M) so that changes in response size would be possible at different temperatures. The concentration of theophylline was also reduced to 10^{-4} M since in the presence of the higher concentration, the tissues were predisposed to a greater incidence of arrhythmias.

The positive inotropic and chronotropic responses of the left and right atria, measured as the increase in tension and rate, respectively, were determined at 42.5, 40, 37.5, 35, 30, 25 and 22.5°C. The rate responses are compared at different temperatures with the increase in cAMP of the right atria in Fig. 5. Both have their optima

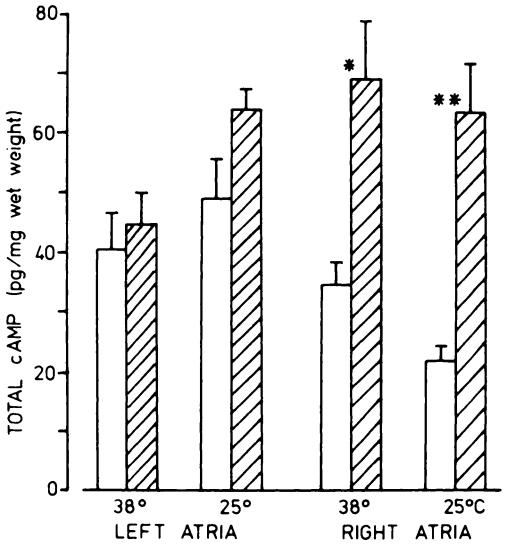

FIG. 3. *Effect of theophylline upon cAMP levels during responses of left and right guinea-pig atria to orciprenaline*

cAMP levels were determined after removing the atria from the bath at 15 seconds into the response to orciprenaline (1.4×10^{-4} M). Determinations were made at 38 and 25°C in the absence (open histogram) or presence (hatched histogram) of theophylline (10^{-3} M). Mean values (± SEM) of at least four experiments are shown. Significance levels for differences between untreated and theophylline-treated atria as calculated by Student's *t*-test are depicted as * $p < 0.05$ and ** $p < 0.02$.

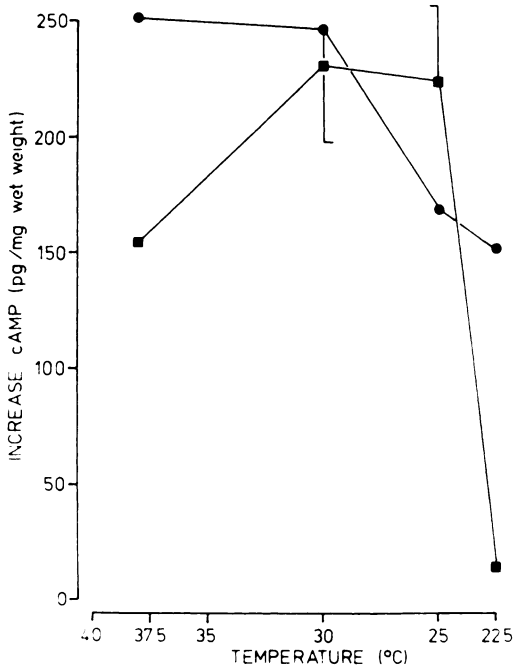

FIG. 4. *Effect of temperature upon the increases in cAMP in the left (■) and right (●) guinea-pig isolated atria in response to orciprenaline*

Determinations were made at 15 seconds into the response to orciprenaline (1.4×10^{-4} M) in the presence of theophylline (10^{-3} M). Each represents the mean of at least four preparations. Vertical bars represent the SEM of that point on the curve.

at or above 37.5°C. The increase tension response and increase cAMP levels of the left atria are plotted against temperature in Fig. 6. Both curves ran parallel and reached their optima at 30°C. When total tension and total cAMP levels (per gram of tissue) of the left atria were plotted (Fig. 7), the optima again coincided, but at 25°C.

DISCUSSION

Elevated levels of cAMP have been demonstrated in guinea-pig left and right atria accompanying the positive inotropic and chronotropic responses to the sympathomimetic amine orciprenaline. This agrees with numerous findings using other β-adrenoceptor agonists such as epinephrine, norepinephrine, and isoproterenol (see 1–4, 22, 23). the cAMP levels were determined separately in whole left and right atria. The changes in cAMP levels of these small masses of tissue were low and only small inconsistent increases were found when the lower submaximal concentration of orciprenaline was used initially. Indeed, this has been the experience of others using different agonists (24, 25) and inotropic responses have been obtained without detectable changes in cAMP (8, 26). However, when a concentration of orciprenaline in excess of that required for maximal mechanical responses was used, the assay procedure was capable of detecting more marked and reproducible increases in cAMP levels.

These changes in cAMP were of the same order in the left and right atria at 38°C. The rate of onset of cAMP production differed, however, that in the right being slower and reaching its peak at 30 seconds compared with 15 seconds for the left atria. The time courses for cAMP production preceded the onset of the physiological responses in both tissues. This agrees with

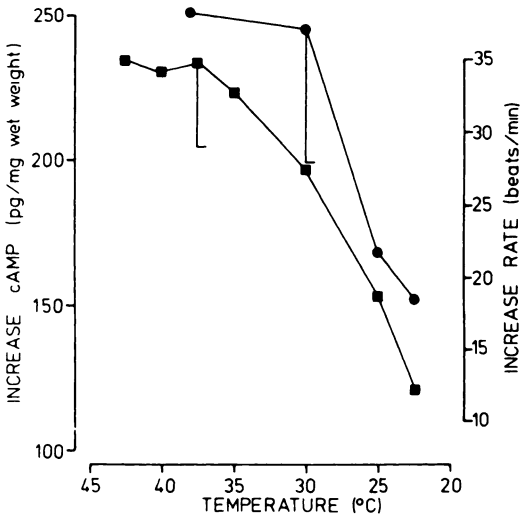

FIG. 5. *Comparison of the effect of temperature upon the increase cAMP (●) and increase rate (■) of guinea-pig isolated right atria in response to orciprenaline*

Mean (n = at least 4) increase in cAMP levels were determined at 15 seconds into the response to orciprenaline (1.4×10^{-4} M) in the presence of theophylline (10^{-3} M). The mean (n = 5) rate increase was measured in separate experiments in which responses to orciprenaline (9.4×10^{-8} M) were allowed to develop fully in the presence of theophylline (10^{-4} M). Vertical bars represent the SEM of that point on the curve.

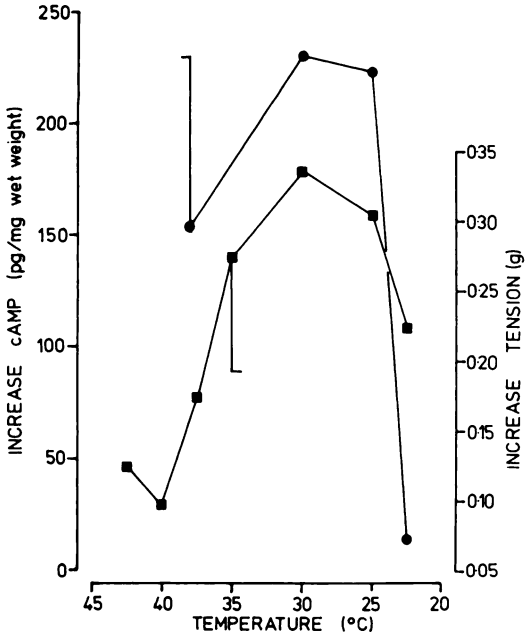

FIG. 6. *Comparison of the effect of temperature upon the increase cAMP* (●) *and increase tension* (■) *of guinea-pig isolated left atria in response to orciprenaline*

cAMP levels are plotted as the mean (n = at least 4) increase determined at 15 seconds into the response to orciprenaline (1.4×10^{-4} M) in the presence of theophylline (10^{-3} M). Tension responses are plotted as the mean (n = 5) increase in developed tension recorded in separate experiments in which responses to orciprenaline (9.4×10^{-8} M) were allowed to develop fully in the presence of theophylline (10^{-4} M). Vertical bars represent the SEM of that point on the curve.

previous observations in isolated hearts (27–30) and atria (24, 31) of several species.

Cooling to 25°C slowed down the rate of onset of the rate and tension responses. This was presumably a post-receptor effect on the electrochemical and contractile events leading to the respective responses (32–34). The rate of cAMP production, however, was not slowed by cooling, the peaks being attained at the same times as at 38°C. Since the time course of cAMP production was the same at both temperatures, the time of sampling was kept constant in the study of the effects of temperature on cAMP levels.

The effects of temperature were examined in the presence of the phosphodiesterase inhibitor theophylline which was shown to enhance the total cAMP levels during the orciprenaline response, significantly in the right atria. This potentiation occurred at both 38 and 25°C. Therefore, although cooling may have inhibited the enzyme to some extent, this was not complete since further potentiation by theophylline was apparently possible, particularly in the right atrium. It must be conceded, however, that theophylline may enhance cAMP levels also by an effect on Ca^{2+} fluxes which may stimulate adenylate cyclase (35). Nevertheless, the hypothermia-induced supersensitivity to sympathomimetic amines (9, 10, 16, 17) is unlikely to be due to phosphodiesterase inhibition by cooling since the inotropic responses to orciprenaline have been shown here and elsewhere (15) to still exhibit supersensitivity in the presence of theophylline.

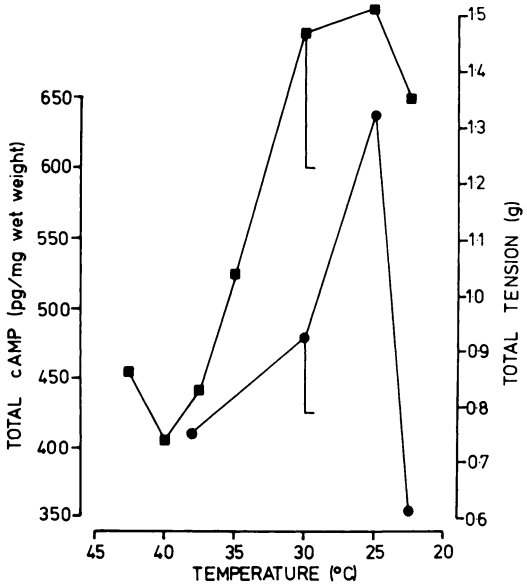

FIG. 7. *Comparison of the effect of temperature upon the total cAMP* (●) *and total tension* (■) *of guinea-pig isolated left atria in response to orciprenaline*

cAMP levels are plotted as the mean (n = at least 4) total value (pg/mg) determined at 15 seconds into the response to orciprenaline (1.4×10^{-4} M) in the presence of theophylline (10^{-3} M). Tension responses are plotted as the mean (n = 5) total developed tension recorded in separate experiments in which responses to orciprenaline (9.5×10^{-8} M) were allowed to develop fully in the presence of theophylline (10^{-4} M). Vertical bars represent the SEM of that point on the curve.

The primary objective of this study was to determine whether the supersensitivity of the inotropic responses was due to a greater stimulation of adenylate cyclase leading to an enhanced production of cAMP. In the left atria, which produce only inotropic responses, the increases in cAMP levels were raised by cooling to 30 and 25°C. The optimum temperature for increased cAMP was the same as for the increase tension response. When both the tension response and cAMP production were measured as total values they also had the same optima. The two effects are therefore modified in parallel by temperature, suggesting that the temperature dependence of the inotropic responses is related to the increased production of cAMP. This also serves as indirect evidence that the accumulation of cAMP plays an obligatory role in the inotropic responses mediated via β-adrenoceptors.

The rate responses were recorded from the right atria, the cAMP levels of which are more difficult to interpret since these atria also produce tension changes. The orciprenaline-induced cAMP production contrasted with that of the left atria, in that cooling did not increase the production. In fact, it fell at 25 and 22.5°C. This closely followed the pattern of the rate responses which also exhibited subsensitivity upon cooling below 37.5°C. The contrast between the subsensitivity of the chronotropic responses and the supersensitivity of the inotropic responses to single submaximal doses of sympathomimetic amines confirms previous studies (10, 16, 17). The lack of hypothermia-induced supersensitivity of the rate responses has also been demonstrated by plotting dose-response curves to the COMT-immune amines (12, 13, 15).

The levels of cAMP measured in the right atria were presumably associated with both rate and tension responses of these preparations. The cells of the SA-node would provide only a small fraction of the cAMP measured, which would be responsible for initiating the rate changes. It is conceivable that in addition to tension changes of the right atrial cells being attributed to increased levels of cAMP, the rate changes of these cells may also be associated with increased cAMP levels per unit time. This argument is favored by the reported phasic changes of cAMP during the cardiac cycle (36). If the cAMP changes producing the tension responses are considered to be enhanced by cooling, as in the left atria, then allowance for this would yield an even greater fall in cAMP production with cooling. In other words, the results represent a substantial overestimate of the actual cAMP levels that contribute to the rate changes. Only by measuring levels in the SA-node could this be satisfactorily answered. The results nevertheless contrast with the left atria and show a close relationship between cAMP production and the rate responses. This, in common with the inotropic responses, supports the concept that positive chronotropic responses due to β-adrenoceptor stimulation are mediated via production of cAMP. The opposing effects of temperature upon the positive inotropic and chronotropic responses and the associated cAMP production might suggest that the β_1-adrenoceptors mediating these responses differ. From pharmacological evidence using agonists and antagonists there is both support for (37, 38) and opposition to (39–41) this suggestion. However, even though the receptors may be pharmacologically indistinguishable, temperature may nevertheless modify the adenylate cyclase or adjacent structures differently in the left and right atria.

The present study has been concerned with the modification by temperature of responses mediated via the β-adrenoceptors of the heart. However, lowering the temperature has been alleged to produce a transformation from β- to α-adrenoceptors in the hearts of frogs (42) and rats (43, 44). For example, the expected antagonism of the positive inotropic responses of rat atria to noradrenaline by propranolol seen at 31°C was no longer evident at 17°C. Instead they were antagonized by the α-adrenoceptor antagonist phenoxybenzamine. However, the cAMP accumulation of rat heart slices was still antagonized by propranolol at the lower temperatures (45). The cAMP and mechanical responses were thus dissociated, which at first sight contradicts the

conclusions from the present study. A similar discrepancy between cAMP production at different temperatures has also been reported with membrane fractions from rat hearts (46). A possible explanation was offered by the finding that cGMP levels increase at lower temperatures which could result from α-adrenoceptor stimulation (24). However, these findings have recently been refuted by Martinez and McNeill (47) who found no dissociation in rat atria on cooling and the theory of receptor transformation would now appear to be open to dispute and based upon dubious data (48). Furthermore, in the guinea-pig atria used here, there is no evidence of α-adrenoceptor activation at any temperature. This conclusion is based upon the facts that phentolamine and phenoxybenzamine fail to antagonize the responses to orciprenaline and isoprenaline at 25°C (15) and blockade by practolol is unaltered by lowering the temperature (9).

If the association between cAMP and the β-adrenoceptor demonstrated here and by many other studies (see 1-4) is accepted, then the supersensitivity of the positive inotropic responses of guinea-pig atria to sympathomimetic amines would appear to be located at the β-adrenoceptor. Indeed, the supersensitivity is selective for sympathomimetic amines (15). An increased affinity for the β-adrenoceptor is an unlikely possibility since the affinity of antagonists, measured as the pA_2 value, is unchanged by cooling (9, 49). The supersensitivity may arise from temperature-dependent steps involving the availability of the receptor or linking receptor occupation with the production of cAMP.

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