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Temperature-induced changes in dissociation constants (K_A) of agonists at cardiac β-adrenoceptors determined by use of the irreversible antagonist Ro 03–7894

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1 The positive inotropic responses of guinea-pig left atria and papillary muscles and positive chronotropic responses of right atria to sympathomimetic amines were examined at 38° and 30°C.

2 At the lower temperature, supersensitivity to orciprenaline and isoprenaline was exhibited as shifts of the dose-response curves to the left and significant reductions in EC_{50} values.

3 This supersensitivity could not be attributed to reduced metabolism since the experiments were performed in the presence of metanephrine (10^{-5}M) and U-0521 (3′,4′-dihydroxy-2-methylpropiophenone) (10^{-4}M) as inhibitors of extraneuronal uptake and catechol-*O*-methyltransferase (COMT) respectively, and the agonists are not susceptible to neuronal uptake.

4 After incubation of the tissues with Ro 03–7894 (1-(5-chloracetylaminobenzfuran-2-yl)-2-isopropylaminoethanol), followed by its prolonged washout (> 2h), the maximum responses to isoprenaline and orciprenaline were depressed, confirming the apparently irreversible β-adrenoceptor antagonism.

5 Dissociation constants (K_A) for isoprenaline and orciprenaline were determined from the equiactive concentrations obtained before (A) and after (A′) incubation with Ro 03–7894, plotted as 1/A against 1/A′ (K_A = (slope − 1)/intercept).

6 K_A values were the same for orciprenaline in the three cardiac preparations and for isoprenaline in the atria. This applied at 38° and 30°C and indicates that the β-adrenoceptors mediating the inotropic and chronotropic responses of the guinea-pig heart do not differ.

7 The K_A values of both agonists were, however, consistently and significantly lower at 30° than at 38°C, indicating an increase in affinity.

8 It is concluded that hypothermia-induced supersensitivity of cardiac tissue to sympathomimetic amines is associated with an increase in their affinity for the β-adrenoceptors.

Introduction

The β_1-adrenoceptor-mediated positive inotropic and chronotropic responses of the heart can be modified by changes in temperature (Broadley, 1980). Cooling of guinea-pig isolated atria causes a leftwards displacement of dose-response curves to sympathomimetic amines, indicative of a supersensitivity (Reinhardt, Wagner & Schümann, 1972; Mori, Hashimoto, Hasegawa & Nakashima, 1979), which appears to be selective for β-adrenocptor agonists since it does not occur with histamine, ouabain or calcium ions (Broadley & Duncan, 1977; Duncan & Broadley, 1978). The hypothermia-induced supersensitivity to isoprenaline is less when examined in the presence of the catechol-*O*-methyl transferase (COMT) inhibitor, tropolone (Broadley & Duncan, 1977) and is less when orciprenaline, a sympathomimetic amine immune to COMT degradation, is used (Reinhard *et al.*, 1972; O'Donnell & Wanstall, 1974; Broadley & Duncan, 1977). Thus, although part of the supersensitivity is due to a reduced activity of COMT at the lower temperature, the remaining supersensitivity can be regarded as specific for the β-adrenoceptor since inhibition of monoamine oxidase (Oppermann, Ryan & Haavik, 1972), phosphodiesterase (Broadley & Duncan, 1977), neuronal uptake (Oppermann, Ryan & Haavik, 1971) and extraneuronal uptake (Wöppel & Trendelenburg, 1973) by cooling have all been exc-

luded as possible explanations for the enhanced sensitivity.

Since there is no change in the affinity of β-adrenoceptor antagonists, as measured by pA_2 values, when temperature is lowered (Reinhardt *et al.*, 1972), it would appear unlikely that an increase in the affinity of sympathomimetic amines could account for their change in potency with hypothermia. However, it has recently been shown that the kinetics of agonist and antagonist binding are different (Weiland, Minneman & Molinoff, 1979; 1980) and the possibility that the supersensitivity may be the result of an increase in affinity of agonists only, must be considered. With the availability of a β-adrenoceptor antagonist, Ro 03-7894 (1-(5-chloracetylaminobenzfuran-2-yl)-2-isopropylaminoethanol), which has been shown to behave in an apparently irreversible manner (Nicholson & Broadley, 1978; Nicholson, Broadley, Burden & Natoff, 1979; Rankin & Broadley, 1982), it has been possible to determine pharmacologically the affinities of sympathomimetic amines for cardiac β-adrenoceptors (Broadley & Nicholson, 1981; Siegl & McNeill, 1982). In the present study, the affinities of isoprenaline and orciprenaline were determined as their dissociation constants, in three cardiac preparations of the guinea-pig, to assess whether changes occur during hypothermia-induced supersensitivity.

A preliminary account of this work was presented to the joint meeting of the British and Scandinavian Pharmacological Societies in Stockholm, July 1982 (Broadley & Williams, 1982).

Methods

Isolated cardiac preparations

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 and mounted on a combined tissue holder and electrode as described previously (Broadley & Lumley, 1977). The left ventricle was cut open and papillary muscles removed, after attaching a cotton loop to the apical end by which they were secured to the tissue holder and then passed through bipolar ring electrodes. The tissues were suspended in 50 ml organ baths 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, $KH_2PO_4 2H_2O$ 1.2) gassed with 5% CO_2 in O_2.

Each tissue was connected to a transducer (Devices type UF1, 57 g sensitivity range) by a cotton thread, for papillary muscles attached to the mitral ends, and the isometric tension recorded on a Devices M19 polygraph. Initial diastolic tensions of approximately 0.5 g were applied to the right atrium and 0.8 g to the left atrium and papillary muscles. Positive inotropic responses were obtained from left atria and paced at a constant rate of 2 Hz with square-wave pulses of 5 ms duration and a voltage of 50% above threshold, delivered by SRI stimulators (type 6053). Positive chronotropic responses were recorded by means of a ratemeter (Devices 2751) triggered by the tension signal of the spontaneously beating right atria. Experiments were carried out at a bath temperature of either 38°C or 30°C maintained by a Churchill chiller circulator (CH/CTC/4).

Drug administration

To determine the dissociation constant (K_A), an equilibrium period of 20 min was allowed with several changes of bathing medium. When the rate and tension were constant, the bathing medium was replaced by one containing metanephrine (10^{-5}M) and U-0521 (10^{-4}M). The tissues were incubated for 20 min before a cumulative dose-response curve to either isoprenaline or orciprenaline was constructed by approximately 3 fold increments in concentration. After restoring the resting rate and tensions to their pre-agonist levels by washing with normal Krebs-bicarbonate solution, the tissues were incubated with Ro 03-7894 (7.6×10^{-4}M) for 30 min. The antagonist was removed from the baths by washing every 20 min for 2 h 40 min, the bathing medium then being changed for one containing metanephrine (10^{-5}M) and U-0521 (10^{-4}M) for 20 min before a final cumulative dose-response curve to the agonist was constructed.

Plotting dose-response curves

Responses were measured as the total rate and total developed tension at each concentration of agonist. Changes in sensitivity of the preparations occurring during an experiment were corrected for by performing control experiments which were identical except that no antagonist was added between the dose-response curves to either isoprenaline or orciprenaline. Mean (n= 4) total rates and total tensions at each concentration on the second curve of control experiments (corresponding to post-antagonist curves) were expressed as a fraction of the total values obtained in the first curve. These factors were then applied to the responses at the respective concentrations in the individual pre-antagonist curves of test experiments. Increases in rate and tension were obtained by subtracting from total values, the resting levels immediately preceding dose-response curves (corrected total and resting levels for pre-antagonist curves). The increases in rate and tension for both

curves were plotted as a percentage of the maximum possible increase. This was calculated by subtracting the resting levels prior to each dose-response curve (corrected value for pre-antagonist curves) from the corrected pre-antagonist maximum total rate or tension. This method of expressing the results avoids possible misinterpretation arising from any change in the resting levels induced by the antagonist.

EC_{50} values for isoprenaline and orciprenaline were determined from individual *uncorrected* pre-antagonist dose-response curves in which the increases in rate or tension were plotted as a percentage of the maximum response. The EC_{50} values were determined by linear interpolation between data points on either side of the 50% response and geometric mean EC_{50} values calculated at 38°C and 30°C. Their 95% confidence limits were calculated as the product of the s.e.mean and the *t* value at the 95% probability level and appropriate degrees of freedom. Statistical analysis was performed throughout by means of Student's *t* test.

Calculation of dissociation constants (K_A)

Dissociation constants were calculated by the method derived by Furchgott for irreversible antagonists (Furchgott, 1966; Furchgott & Bursztyn, 1967; Besse & Furchgott, 1976). Individual pre- and post-antagonist dose-response curves were plotted as described above by linear interpolation between points and the following equation applied:

$$1/(A) = (1-q)/q(K_A) + {}^1/q(A')$$

where q is the fraction of receptors remaining unoccupied by the irreversible antagonist Ro 03-7894. Equiactive molar concentrations of agonist obtained before (A) and after washout (A') of Ro 03-7894 were determined. The reciprocal values were plotted as $^1/A$ against $^1/A'$ and from the calculated regression line, the dissociation constant was determined (K_A = (slope − 1)/intercept on the $^1/A$ axis).

Drugs

The antagonist Ro 03-7894 (1-(5-chloracetylaminobenzfuran-2-yl)-2-isopropylaminoethanol) was synthesized in the chemistry laboratories of Roche Products Ltd., Welwyn Garden City, Herts, England. (−)-Isoprenaline tartrate dihydrate (Ward Blenkinsop Ltd.), (±)-orciprenaline sulphate (Boehringer Ingelheim) and U-0521 (3',4'-dihydroxy-2-methylpropiophenone) (The Upjohn Company) were kindly supplied as gifts. (±)-Metanephrine hydrochloride was obtained commercially (Sigma). All solutions were freshly prepared in distilled water. Ascorbic acid (1 μg ml^{-1}) was added to the isoprenaline and orciprenaline solutions and to the Ro 03-7894 (1 mg ml^{-1}) to aid solution.

Results

Effect of temperature upon sensitivity to isoprenaline and orciprenaline

Lowering the bath temperature from 38° to 30°C produced supersensitivity of isolated atria as shown by the leftwards displacement of the dose-response curves for the positive inotropic and chronotropic responses to isoprenaline (Figure 1a) and orciprenaline (Figure 1b) of left and right atria respectively, and for the positive inotropic responses of papillary muscles to orciprenaline (Figure 1c). The mean EC_{50} values ($n > 8$) of orciprenaline at 30°C on right and left atria and papillary muscles of

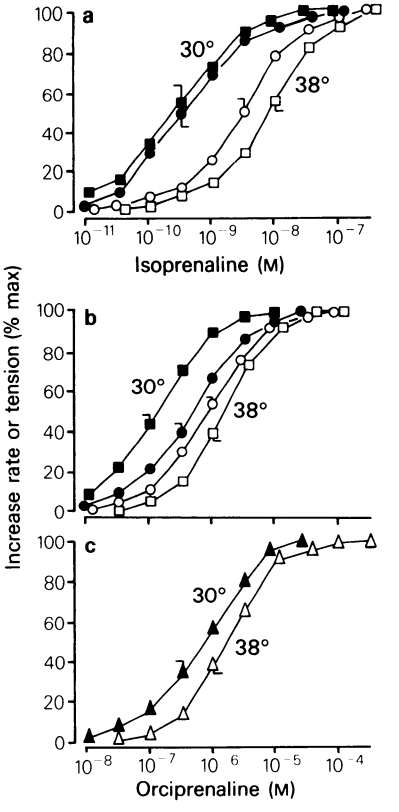

Figure 1 Effect of temperature on the mean ($n > 8$) cumulative dose-response curves for isoprenaline and orciprenaline on guinea-pig left and right atria (a and b) and papillary muscles (c). Positive inotropic responses of left atria (□■) and papillary muscles (△▲) and positive chronotropic responses of right atria (○●) were obtained at 38° (open symbols) and 30°C (solid symbols).

0.37 (0.25–0.55), 0.19 (0.10–0.37) and 0.69 (0.38–1.23) μM respectively were all significantly ($P<0.01$) lower than the corresponding values of 1.01 (0.74–1.37), 1.36 (1.02–1.82) and 2.16 (1.46–3.2) μM obtained at 38°C. Similarly, for isoprenaline the mean EC_{50} values ($n>8$) at 30°C on the left (0.31 (0.15–0.65)nM) and right atria (0.38 (0.20–0.71)nM) were significantly ($P<0.001$) less than the values at 38°C (7.16 (4.0–13.0) and 3.3 (1.8–6.0)nM respectively).

When absolute rate and tension values were measured, the resting rate of right atria fell significantly ($P<0.001$) from 247.9±6.2 beats min^{-1} at 38°C to 171.4±5.3 beats min^{-1} at 30°C (combined isoprenaline and orciprenaline data), and the maximum developed rate was significantly ($P<0.001$) reduced by lowering the temperature from 332.6±25.0 to 210.0±14.0 beats min^{-1} for orciprenaline and from 367.8±6.5 to 222.0±4.1 beats min^{-1} for isoprenaline. However, lowering the temperature resulted in a significant ($P<0.05$) increase in the resting developed tensions of left atria and papillary muscles from 0.37±0.06 and 0.19±0.03 g respectively at 38°C to 0.79±0.10 and 0.51±0.07 g at 30°C. The maximum developed tensions of these tissues in response to either agonist did not alter significantly ($P>0.05$) with changes in temperature.

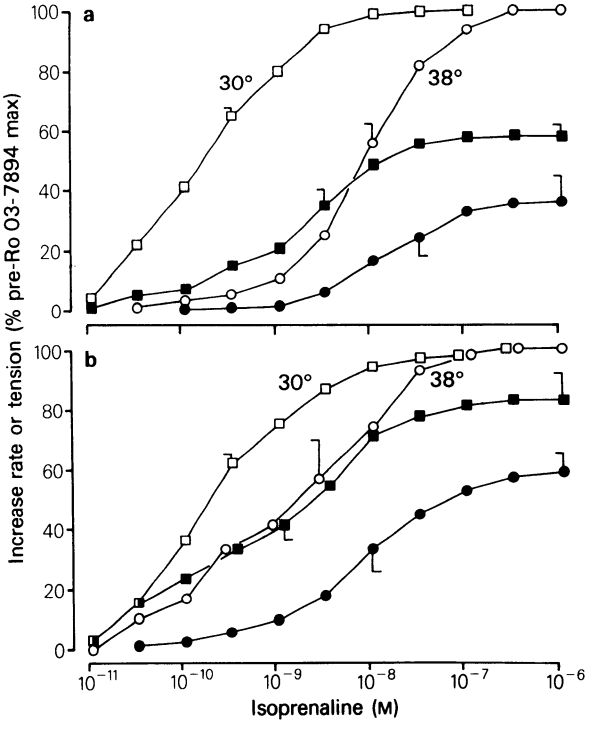

Figure 2 Effect of Ro 03–7894 on mean ($n>5$) cumulative dose-response curves for isoprenaline on (a) left atria and (b) right atria of guinea-pigs. Positive inotropic responses of left atria and positive chronotropic responses of right atria were obtained before (open symbols) and after (solid symbols) incubation with Ro 03–7894 (7.6×10^{-4}M), followed by its washout. Experiments were performed at either 38°C (○) or 30°C (□). Pre-antagonist curves were corrected from controls as described in the text.

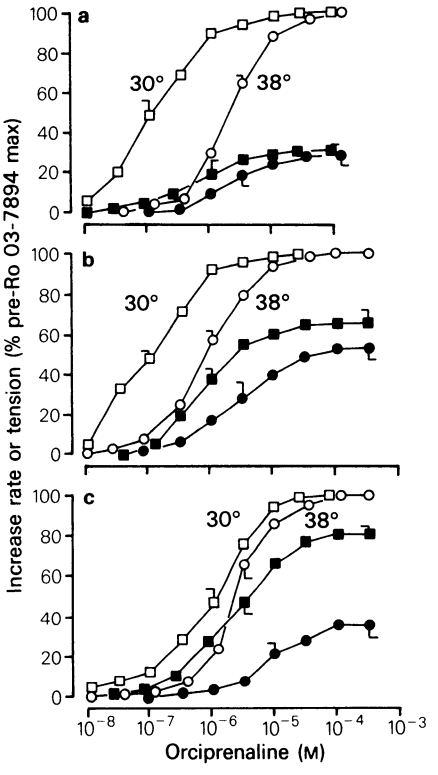

Figure 3 Effect of Ro 03–7894 on mean ($n>4$) cumulative dose-response curves for orciprenaline on (a) left atria, (b) right atria and (c) papillary muscles of guinea-pigs. Positive inotropic responses of left atria and papillary muscles and positive chronotropic responses of right atria were obtained before (open symbols) and after (solid symbols) incubation with Ro 03–7894 (7.6×10^{-4}M), followed by its washout. Experiments were performed at either 38°C (○) or 30°C (□). Pre-antagonist curves were corrected from controls as described in the text.

Antagonism by Ro 03–7894

After incubation of the tissues with Ro 03–7894 (7.6×10^{-4}M), followed by its washout, the maximum responses to isoprenaline (Figure 2) and orciprenaline (Figure 3) were significantly ($P<0.05$) depressed in all three cardiac preparations. This occur-

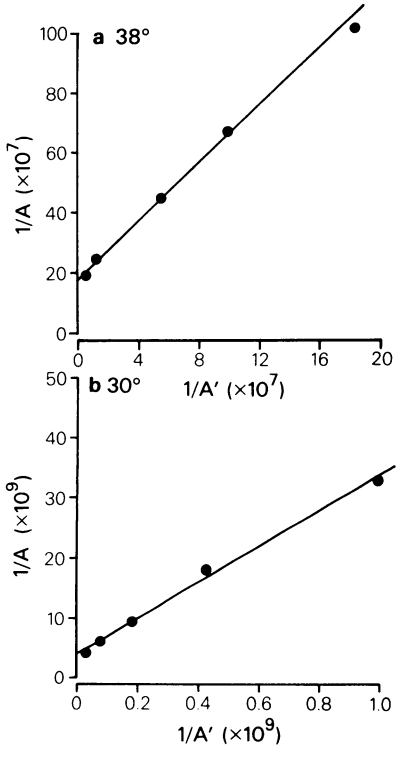

Figure 4 Graphs of reciprocals of molar concentrations of isoprenaline obtained before Ro 03–7894 (1/A) plotted against reciprocals of equiactive molar concentrations after incubation with Ro 03–7894 (1/A'). These values were obtained from the mean cumulative dose-response curves for the positive inotropic responses of left atria to isoprenaline at (a) 38°C ($n = 8$) and (b) 30°C ($n = 5$), shown in Figure 2a.

red at both 38° and 30°C, and the depression of the maxima by Ro 03–7894 was generally less at 30° than at 38°C. However, the difference was significant only for isoprenaline on left atria ($P < 0.05$, Figure 2a) where the maximum tension increase was depressed to 37.1 ± 7.7% at 38°C and 58.1 ± 3.0% at 30°C; and for orciprenaline on papillary muscles ($P < 0.001$, Figure 3c) where the maximum response was depressed to 35.0 ± 6.6% at 38°C and 80.0 ± 2.8% at 30°C.

Dissociation constants (K_A)

Dissociation constants were calculated from the dose-response curves obtained before and after incubation with Ro 03–7894. Graphs of the reciprocals of equiactive molar concentrations of agonist obtained before (1/A) and after Ro 03–7894 (1/A') were plotted and typical graphs from the mean dose-response curves to isoprenaline in left atria at 38 and 30°C are shown in Figure 4. The mean K_A values (± s.e.mean) for isoprenaline and orciprenaline determined from individual experiments are shown in Table 1.

The dissociation constants for isoprenaline were greater than for orciprenaline. The dissociation constants obtained for isoprenaline did not differ significantly ($P > 0.05$) between the left and right atria and for orciprenaline there was no significant ($P > 0.05$) difference between the three preparations. This applied at both 38° and 30°C. However, when the K_A values were compared between the two temperatures, the values obtained at 30°C were significantly ($P < 0.05$) less than those obtained at 38°C. The decrease in K_A value occurred with both agonists in each preparation examined and was approximately 4 fold.

Discussion

A reduction of the bathing temperature of guinea-pig isolated atria and papillary muscles resulted in an enhanced sensitivity to the β-adrenoceptor agonists, isoprenaline and orciprenaline. This supersensitivity was measured as a decrease in the EC_{50} values for the positive inotropic and chronotropic responses and

Table 1 Dissociation constants (K_A) of isoprenaline (nM) and orciprenaline (μM) on guinea-pig cardiac tissues at 38 and 30°C

	Isoprenaline		*Orciprenaline*	
Tissue	38°C	30°C	38°C	30°C
Left atria	28.0 ± 6.9 ($n = 8$)	*5.7 ± 2.4 ($n = 5$)	4.3 ± 1.3 ($n = 4$)	*0.96 ± 0.47 ($n = 4$)
Right atria	37.7 ± 14.5 ($n = 5$)	*4.5 ± 1.5 ($n = 5$)	8.4 ± 2.5 ($n = 6$)	*2.3 ± 1.1 ($n = 5$)
Papillary muscles	—	—	7.8 ± 1.3 ($n = 4$)	*3.0 ± 1.2 ($n = 4$)

K_A values are the mean ± s.e.mean. Values at 38 and 30°C were significantly different (*$P < 0.05$), as determined by Student's t test.

confirms numerous previous observations (see Broadley, 1980 for review). The demonstration of hypothermia-induced supersensitivity of the positive chronotropic responses of guinea-pig right atria depends upon the method of expressing the results (Broadley, 1974). Although the resting rate and maximum developed rate were both reduced by hypothermia, when the increases in rate were expressed as a percentage of the maximum at each temperature, supersensitivity was evident from the fall in EC_{50} value at the lower temperature. Since there were no changes in absolute tension values of left atria and papillary muscles, the supersensitivity of these tissues was considered to be independent of the method of plotting the data.

The hypothermia-induced supersensitivity was demonstrated in the presence of U-0521 and metanephrine as inhibitors of COMT (Bönisch & Uhlig, 1973) and of extraneuronal uptake respectively. These metabolic factors together with inhibition of monoamine oxidase (Oppermann *et al.*, 1972) and neuronal uptake (Oppermann *et al.*, 1971) by cooling can therefore be discounted as explanations for the supersensitivity. It has previously been shown to be specific for β-adrenoceptor agonists (Broadley & Duncan, 1977; Duncan & Broadley, 1978) and the purpose of the present study was to determine by pharmacological means whether the enhanced sensitivity is due to a change in the affinity of the agonist for the cardiac β-adrenoceptor. The dissociation constants of isoprenaline and orciprenaline were determined by use of the irreversible β-adrenoceptor antagonist Ro 03-7894 (Nicholson & Broadley, 1978; Nicholson *et al.*, 1979; Rankin & Broadley, 1982). The apparently irreversible nature of the antagonism was confirmed by the depression of maximum responses even after prolonged (> 2 h) washout of the antagonist. The depression of maximum responses was generally less at the lower temperature, confirming earlier observations (Broadley & Nicholson, 1980) and reflecting the enhanced receptor sensitivity at the lower temperature. Dissociation constants were calculated only after thorough washout of free Ro 03-7894 since any residual antagonist exerts an additional competitive antagonism which distorts the K_A value (Broadley & Nicholson, 1981). Furthermore, care was taken to measure equiactive concentrations on the linear portions of pre- and post-Ro 03-7894 dose-response curves (see Thron, 1970).

The dissociation constants of isoprenaline and orciprenaline were consistently reduced at the lower temperature, indicating an increase in affinity of agonist binding for the receptor. This finding would seem at variance with that obtained with β-adrenoceptor antagonists. The affinities of antagonists, measured as the pA_2 value, have been shown to remain unaltered at different temperatures in guinea-pig (Reinhardt *et al.*, 1972) and mouse atria (Muñoz-Ramírez, Ryan & Buckner, 1975). This anomaly may be explained by observations from β-adrenoceptor binding studies, which indicate a difference in the interaction of agonists and antagonists with the β-adrenoceptor (Williams & Lefkowitz, 1977). Agonists are thought to bind to a low affinity state of the receptor inducing conformational changes to a high affinity state, whereas antagonists are unable to induce this transition (Kent, DeLean & Lefkowitz, 1980). At low temperatures, the affinity of agonists for inhibition of [^{125}I]-iodohydroxybenzylpindolol binding was found to be increased, but the change in affinity of antagonists was less (Weiland *et al.*, 1979, 1980). The affinity of agonist, but not antagonist, binding to β-adrenoceptors in turkey erythrocytes (Pike & Lefkowitz, 1978) and S49 lymphoma cells (Insel & Sanda, 1979) is also increased as the temperature is lowered, although in these experiments the temperature was reduced as far as 4°C. Increases in the affinity of agonist binding brought about by lowered temperatures have also been reported for muscarinic receptors of the rat heart (Phan, Wei & Sulakhe, 1980) and α-adrenoceptors of calf brain (U'Prichard & Snyder, 1978).

The dissociation constants for orciprenaline obtained at 38°C were identical in the three cardiac preparations. Similar values were also obtained for the positive inotropic and chronotropic responses of left and right atria to isoprenaline, as shown previously (Broadley & Nicholson, 1979; 1981). Yet these agonists exhibited a rate-selectivity at 38°C, the EC_{50} values being less for the rate responses, and the possibility has been suggested that the β-adrenoceptors mediating the positive inotropic and chronotropic responses can be distinguished (Dreyer & Offermeier, 1975; Giudicelli, 1975). However, the bulk of pharmacological evidence refutes this proposal (Lumley & Broadley, 1977; Kaumann, McInerny, Gilmour & Blinks, 1980), for the guinea-pig at least, where only β_1-adrenoceptors mediate these responses (Zaagsma, Oudhof, Van der Heijden & Plantjé, 1979). The present finding of similar dissociation constants for both the inotropic and chronotropic responses also supports the view that the β-adrenoceptor recognition sites are identical for these responses.

In conclusion, the present study has shown that the hypothermia-induced supersensitivity to the positive inotropic and chronotropic responses to sympathomimetic amines is associated with an increase in the affinity of agonists for the β-adrenoceptor.

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