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Comparison of the chronotropic effect and the cyclic AMP accumulation induced by β_2-agonists in rat heart cell culture

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1 The chronotropic response and the variation in cyclic adenosine 3',5'-monophosphate (cyclic AMP) accumulation induced by isoprenaline and six β_2-selective agonists (fenoterol, salmefamol, soterenol, zinterol, salbutamol and formoterol) were analyzed on cultured heart cells of the rat.

2 The compounds elicited an enhancement of the frequency, but the time course of the variation of the beating rate was not identical for all of them. A rapid onset was observed for isoprenaline, zinterol and formoterol while it was slower for fenoterol, salmefamol and salbutamol.

3 In contrast with isoprenaline, the β_2-selective agonists gave concentration-beating frequency curves which were not sigmoidal. Their effects extended up to a concentration of 5 to 6 orders of magnitude. Nevertheless, the concentration at which the maximal effect occurred and the intrinsic activities of the various compounds agrees better with the responses observed on guinea-pig atria than with those on trachea.

4 All the β_2-selective agonists increased the accumulation of cyclic AMP in rat heart cells with a maximal effect at 10^{-5} M or less. The effects of β_2-agonists on cyclic AMP production showed some analogies with those on beating frequency of the heart cells. The increase in cyclic AMP accumulation induced by β_2-agonists also corresponded to their chronotropic effects on guinea-pig atria. Thus, the correlation coefficient between the inverse of the log of the concentration producing the half maximal cyclic AMP accumulation in cultured heart cells and the pD_2 values on guinea-pig atria was 0.93.

5 It is concluded that, in contrast to what was observed in other models, the β_2-selective agonists induce an increase in the production of cyclic AMP in rat heart cells. Furthermore, the effects of the β_2-agonists on cyclic AMP accumulation and on beating rate in the heart cells may correspond with their β_1-adrenoceptor potencies.

Introduction

Since the subclassification by Lands, Arnold, McAuliff, Luduena & Brown (1967) of the β-adrenoceptors into β_1 and β_2, the quest for selective β_1 and β_2-antagonists and agonists has continued. Pharmacological assays to assess the selectivity of the compounds generally use guinea-pig atria (β_1) and trachea (β_2) although the β-adrenoceptor population in these tissues may not be completely homogenous (Hedberg, Minneman & Molinoff, 1980; O'Donnell & Wanstall, 1979). The stimulation of heart β-adrenoceptors, which are mainly β_1-subtype, induce positive inotropic as well as positive chronotropic effects. In guinea-pig atria, the increase in adenosine 3',5'-cyclic monophosphate (cyclic AMP) level produced by isoprenaline has been shown to parallel its inotropic response (Osnes & Øyes, 1975). For the chronotropic effect, the relation between response and cyclic AMP level is not so clearly established although some evidence on cultured heart cells supports this view (Wollenberger & Irmler, 1978).

β_2-Selective agonists, despite a lower potency on heart than on trachea, still have a considerable chronotropic effect on guinea-pig atria (Decker, Quennedey, Rouot, Schwartz & Velly, 1982). However, contrary to what is observed with isoprenaline, no significant increase in cyclic AMP level could be detected in atria in the presence of the β_2-selective agonists, soterenol and terbutaline (Buckner, Torphy

& Costa, 1978; Hedberg, Carlsson, Fellenius & Lundgren, 1982). Furthermore, Minneman, Hegstrand & Molinoff (1979) reported that β_2-selective drugs such as soterenol, zinterol, terbutaline and salbutamol, did not stimulate adenylate cyclase in rat ventricle homogenate. Among the β_2-selective agonists tested by these authors, only fenoterol elicited 45% of the maximal stimulation of adenylate cyclase produced by isoprenaline. Thus among β-agonists with positive chronotropic effects it would seem that some do, but others do not, stimulate adenylate cyclase also.

To investigate further the biological and biochemical responses of β_2-agonists, we examined the effects of isoprenaline and six β_2-selective agonists (Table 1) on the beating rate and on the cyclic AMP accumulation of heart cell cultures. These results were compared to earlier pharmacological data obtained with guinea-pig atria and trachea.

Methods

Heart cell cultures

Heart cell cultures were prepared from 3 day old rats (Sprague-Dawley), according to the method of Harary & Farley (1963) with some modifications. Cells were isolated from the minced hearts by trypsinization at 23°C. The enzyme was twice crystallized from pig pancreas (Laboratoires Choay, Paris) and was used at a 0.25–0.50 g/l concentration in Dulbecco's buffered Ca^{2+}- and Mg^{2+}-free salt solution. Repeated 5 min incubations with trypsin were done until the tissue was almost completely dispersed. Leighton's tubes, equipped with removable plastic slides as cell carriers, were used as culture vessels. The cells were plated into the tubes at a density of 6×10^5 in 2.5 ml Eagle's minimum essential medium (MEM) with 10% calf serum and antibiotics. They were grown at 37°C for 6–8 days. The gas phase was air with no additional CO_2, and the pH was adjusted to 7.40 every other day with a 4.2% (w/v) $NaHCO_3$ sterile solution. To limit proliferation of fibroblasts, the medium was not renewed.

Investigation of β-agonist effects

Drugs were dissolved in ascorbic acid (1 mg/ml in distilled water): 25 µl of the drug solution (i.e. 1% of the volume of medium) was added to each tube to give final concentrations ranging from 10^{-10} to 10^{-4} M.

Beating rate was measured at 37°C under an inverted microscope at a magnification of 300 × in 6 randomly selected microscopic fields per tube, at least 3 times before starting the experiment. The values thus obtained were taken as control values. Thirty seconds, 2 min 30 s, 5 min 30 s and 29 min 30 s after the addition of the drug solution, beating rate was observed for 1 min which allowed 4–6 successive measurements.

Spontaneous beating rate varied between cultures and even between tubes within a given culture. In the present work, the lowest value observed was 64, the highest 240 beats/min; however, such extreme values were rare and more than half the values ranged between 130 and 160. The mean was 144 ± 3 beats per min. The changes induced by the β-agonists were expressed as a percentage of the untreated rate.

The β-agonist effects on beating frequency were investigated on 6 individual heart cell cultures comprising an average 75 Leighton tubes, each tube receiving a single dose of one product only.

Determination of intracellular cyclic AMP content

Intracellular cyclic AMP levels were measured on 3 individual heart cell cultures. Assays were carried out at least in duplicate for each drug concentration on each culture. The cells were preincubated for 5 min at 37°C with 0.5 mM of 3-isobutyl-1-methylxanthine (IBMX); 25 µl of the drug solution was added to each Leighton's tube to give final concentrations ranging from 10^{-8} to 10^{-4} M. After a further 90 s incubation at 37°C, the cell loaded slides were immersed in ice-cold 0.1 N perchloric acid, then quickly rinsed with ice-cold saline. They were immediately scraped off with a silicone policeman and transferred into test tubes with two washes of 100 µl of Tris-HCl 100 mM (pH 7.4 at 25°C). Test tubes were centrifuged at 1000 *g* for 15 min.

Supernatants were assayed in duplicate for cyclic AMP content according to Brown, Albano, Ekins & Sgherzi (1971). Pellets were used for protein determination according to Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as a standard. Control tubes were assayed under the same experimental conditions except for the addition of β-agonists. The results were expressed as pmol cyclic AMP mg^{-1} protein.$90\,s^{-1}$.

The protein contents of the culture tubes were very similar within a given culture and not very different between individual heart cell cultures. Conversely, variations in the biochemical responses to β-agonists from one culture to another were more pronounced. For instance, the values for cyclic AMP accumulation induced by 10^{-7} M isoprenaline ranged between 110 and 445 pmol/mg of protein depending on the heart cell preparation. However, the basal levels (controls) were more homogeneous varying from 18.9 to 33.2 pmol/mg of protein with a mean value of 24.7 ± 2.9.

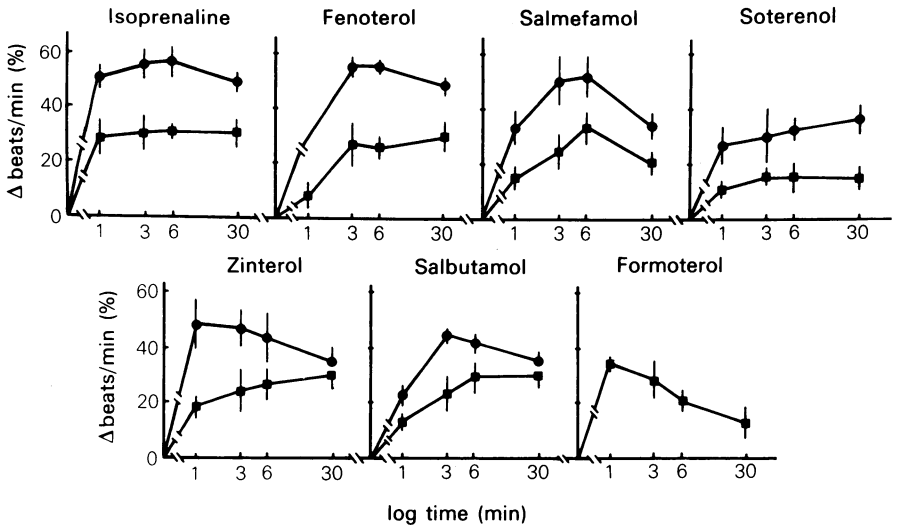


Figure 1 Time course of the effects of the β-agonists on the beating rate of rat heart cells. The rate was measured as described under Methods before and after 1, 3, 6 and 30 min incubation with the various drugs. Each result is the mean ($n = 7-10$, with s.e. indicated by vertical lines) of the percentage increase in frequency at the concentration which produced the maximal (●) and the half maximal (■) effect. Isoprenaline 10^{-8}, 10^{-9}, fenoterol 10^{-5}, 10^{-7}, salmefamol 10^{-5}, 10^{-8}, soterenol 10^{-6}, $\frac{10^{-8}+10^{-7}}{2}$, zinterol 10^{-4}, $\frac{10^{-6}+10^{-7}}{2}$, salbutamol, 10^{-4}, 10^{-6}, formoterol 10^{-8}M.

Drugs

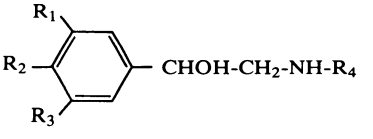
The drugs (for chemical structures see Table 1) were obtained from the following sources: formeterol fumarate, also known as BD40A (Yamanouchi Pharmaceutical Co.), (−)-isoprenaline hydrochloride (Sigma), fenoterol hydrobromide (Boehringer Ingelheim), salbutamol sulphate (Glaxo), salmefamol base (Glaxo), soterenol hydrochloride (Mead-Johnson), zinterol hydrochloride (Mead-Johnson). All other chemicals were of analytical grade.

Results

Time course of effect of β-agonists on heart cell beating rate

All β-agonists investigated induced a positive chronotropic response in heart cell cultures, which was observed for 30 min. Nevertheless, the acceleration in beating rate induced by these drugs did not follow the same pattern. This is shown in Figure 1, where the variations induced by two of the 7 concent-

Table 1 Chemical structures of the series of β-agonists studies



	R_1	R_2	R_3	R_4
Isoprenaline	H	OH	OH	$-CH(CH_3)_2$
Fenoterol	OH	H	OH	$-CH(CH_3)-CH_2-C_6H_4OH(p)$
Salmefamol	H	OH	CH_2OH	$-CH(CH_3)-CH_2-C_6H_4OCH_3(p)$
Soterenol	H	OH	$NHSO_2CH_3$	$-CH(CH_3)_2$
Zinterol	H	OH	$NHSO_2CH_3$	$-C(CH_3)_2-CH_2-C_6H_5$
Salbutamol	H	OH	CH_2OH	$-C(CH_3)_3$
Formoterol (BD-40A)	H	OH	NHCHO	$-CH(CH_3)-CH_2-C_6H_4OCH_3(p)$

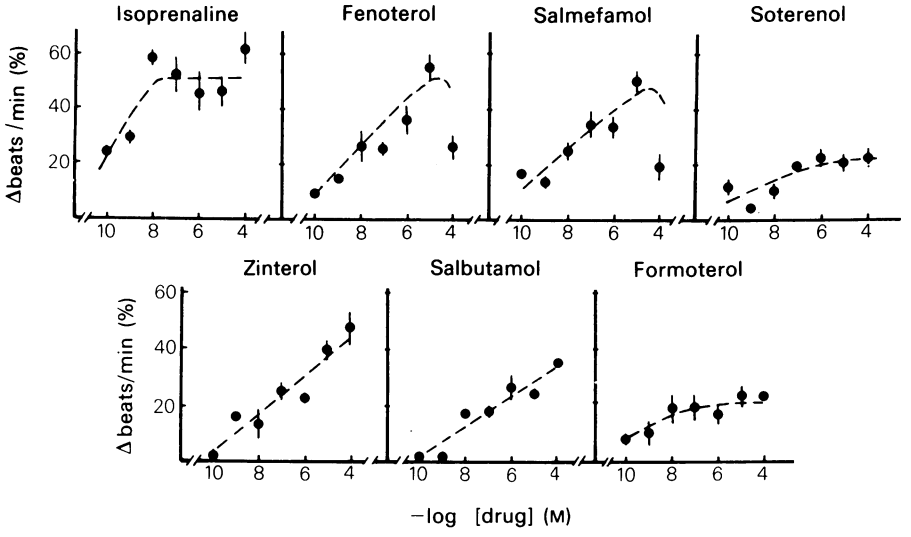


Figure 2 Log concentration-response curves for increases in beating rate of cultured rat heart cells in response to β-agonists. Variations in beating rate are expressed as the mean ($n = 7-10$, s.e. indicated by vertical lines) of the percentage increase in frequency after 3 min incubation with various drugs concentrations.

rations tested, one responsible for the maximal effect, the other for the experimental 50% effect, were plotted as a function of the logarithm of time. The maximal effects of isoprenaline, soterenol, zinterol and formoterol occurred within 1 min. This was not the case for salmefamol, salbutamol and fenoterol which produced their maximal effect between 3 and 6 min; the increase in frequency observed at 1 min was roughly half-maximal. The β-agonists studied here also differed in the duration of the maximal effect. The maximal effect of isoprenaline, fenoterol, soterenol remained unchanged for 30 min. In contrast, the increase in beating rate fell after 6 min for salmefamol and for the highest concentrations of zinterol and salbutamol. Similarly, the effect of formoterol (10^{-8} M) which was maximal at 1 min was followed by a sharp decrease.

Concentration-beating rate response relationship

The β-agonists produced an increase in the beating frequency that was concentration-dependent. The percentage variations in the beating rate 3 min after adding the β-agonist are plotted in Figure 2 as a function of drug concentration. For isoprenaline, the concentration-response curves gave the expected sigmoidal pattern. In contrast, for the other β-agonists, the concentration-response curves showed no such

Table 2 Comparison of the increase in beating rate and cyclic AMP production induced by the β-agonists with their effects on guinea pig trachea and atria

	Guinea-pig[a]				*Heart cells*			
	Trachea (β_2)		Atria (β_1)		Beating rate[b]		Cyclic AMP[c]	
	pD_2	i.a.	pD_2	i.a.	Max effect	i.a.	Log $1/ED_{50}$	i.a.
(−)-Isoprenaline	8.57	1.00	8.62	1.0	10^{-8}	1	8.25	1.00
(+)-Fenoterol	7.61	0.92	6.85	1.05	10^{-5}	0.95	7.00	1.22
(±)-Salmefamol	8.23	0.92	6.78	0.82	10^{-5}	0.87	6.82	1.17
(±)-Salbutamol	7.13	0.91	5.90	0.75	10^{-4}	0.62	6.06	0.79
(±)-Zinterol	8.53	0.91	6.25	0.70	10^{-4}	0.81	7.12	0.95
(±)-Soterenol	7.59	0.90	6.26	0.56	10^{-6}	0.39	6.52	0.68
(±)-Formoterol	9.29	0.94	6.98	0.94	10^{-5}	0.42	7.88	1.11

[a] Data are taken from Decker *et al.* (1982) except those for fenoterol which were given by Dr Decker.
[b] The values refer to the concentration which produced the maximal increase in the beating rate. The intrinsic activities were calculated in relation to the effect of isoprenaline at 10^{-8}M.
[c] ED_{50} values for cyclic AMP accumulation were estimated from the plots of figure 3 as the concentration giving 50% maximal effect. Intrinsic activities were calculated in relation to the effect obtained with 10^{-7}M of isoprenaline.

pattern and the responses extended over concentrations of 5 to 6 orders of magnitude. Under these conditions, it was difficult to estimate ED_{50} values and only the concentrations which produced the maximal response for each drug are summarized in Table 2. However, for zinterol and salbutamol, the concentration-response curves did not level off up to 10^{-4}M; for these drugs, the effect obtained at this concentration was considered as the maximal effect. The intrinsic activity was determined in relation to isoprenaline (10^{-8}M) and here too that for zinterol and salbutamol was estimated from the highest concentration tested on heart cells (i.e. 10^{-4}M). Isoprenaline was the more potent compound since the concentration giving the maximal effect, i.e. 10^{-8}M, was 1000 to 10000 times lower than for the other drugs. Formoterol often had erratic effects on the beating rate of heart cells since periods of high and low frequencies alternated during incubation. A similar phenomenon was also observed with soterenol and salbutamol but only at concentrations of 10^{-5} and 10^{-4}M. Data from such irregular observations were discarded from the results plotted in Figure 2.

Concentration-dependent cyclic AMP production induced by β-adrenoceptor agonists

Intracellular cyclic AMP production was determined after 90 s incubation with the β-agonists since Wollenberger & Irmler (1978) showed that the maximal effect of adrenaline occurred at 30 s and remained constant for at least 3 min in the presence of IBMX.

The mean cyclic AMP content as a function of β-agonist concentrations is plotted in Figure 3. All the compounds increased the intracellular cyclic AMP level. The maximal responses elicited for fenoterol, salmefamol, zinterol and formoterol were close to that obtained with 10^{-7}M isoprenaline. In contrast, salbutamol and soterenol produced respectively 78% and 68% of the maximal cyclic AMP accumulation of isoprenaline. Since the slope of the concentration-cyclic AMP production curves were close to the sigmoïdal pattern expected, ED_{50} values were estimated from the plots of Figure 3 as being the concentrations required for 50% maximal stimulation observed with a particular drug. The values of log $1/ED_{50}$ are summarized in Table 2.

Relationships between the variations in the beating rate and the cyclic AMP accumulation induced by β-agonists on heart cells and with their β_1/β_2 potencies

From the plots of Figures 2 and 3, it appears that the effect on the heart cell beating rate occurred at lower concentrations than those required to produce an increase in intracellular cyclic AMP.

This phenomenon is exemplified by the concentration-response curves of isoprenaline. Thus, at 10^{-8}M, isoprenaline induced about 100% of its

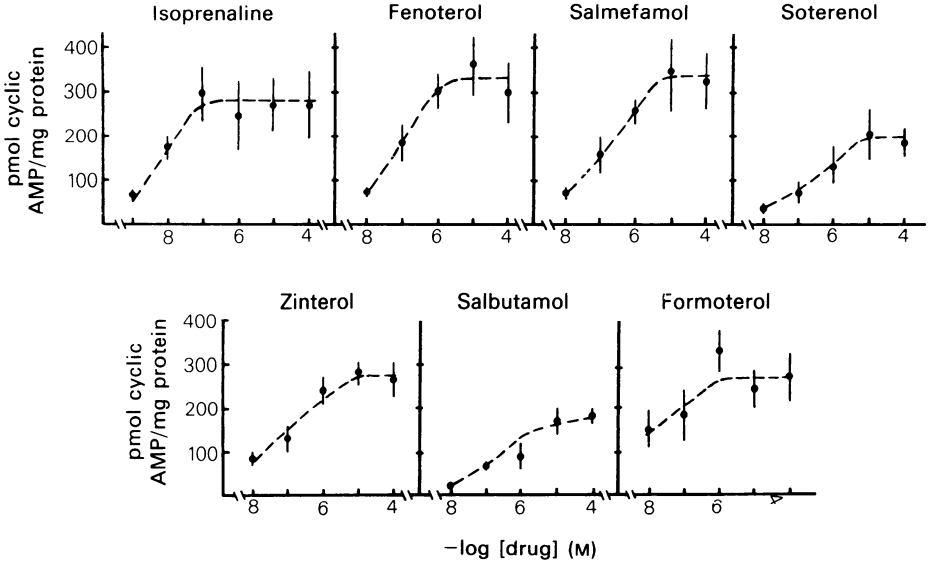


Figure 3 Log concentration-response curves for increases in the cyclic AMP contents of cultured rat heart cells in response to β-agonists. The intracellular cyclic AMP content was measured as described under Methods, after the cells has been preincubated with IBMX for 5 min. Each result is the mean (with s.e. for 6 to 8 determinations shown by vertical lines) of cyclic AMP content, expressed as pmol/mg of protein, after 90 s incubation with the various β-agonists. Mean control value (no drug added) was 24.7 ± 2.9 pmol of cyclic AMP/mg of protein.

maximal enhancement of beating rate whereas cyclic AMP accumulation was only 58%. A similar effect was observed at low concentrations with the other β-agonists except for formoterol. However, strictly parallel concentration-response curves were obtained with isoprenaline while for the other drugs, the two concentration-response curves tended to coincide at high concentrations.

Table 2, which gives pD_2 values previously obtained on guinea-pig atria (β_1) and trachea (β_2) together with the results of the present study, permits some comparisons. Thus, the large difference between the concentrations at which isoprenaline and the other β-agonists induced the maximal increase in heart cell beating rate is also observed between the pD_2 values determined for the chronotropic effect on guinea-pig atria (8.62 for isoprenaline compared with values below 7 for the others). Furthermore, the intrinsic activity of the chronotropic effect on heart cells and isolated atria are clearly connected since the correlation coefficient was 0.88 ($P < 0.05$) when formoterol was omitted. This value was higher than that between the corresponding intrinsic activity in heart cell and trachea ($r = 0.64$).

The same observation holds true when the logarithm of the concentrations producing 50% maximal cyclic AMP accumulation are compared to the pD_2 values for guinea-pig isolated tissues. Thus, the correlation coefficient between log $1/ED_{50}$ and the pD_2 values on atria is 0.93 ($P < 0.01$) while with the trachea pD_2 values, this coefficient is 0.80 ($P < 0.1$), in the absence of formoterol. Similarly, the β-agonist intrinsic activities of cyclic AMP production clearly correspond to those on guinea-pig atria, with a correlation coefficient of 0.79 ($P < 0.05$) compared to 0.29 for intrinsic activities on trachea.

Discussion

The results of this study show that β_2-selective agonists produce both an increase in beating rate and in cyclic AMP production of cultured rat heart cells. The onset of the maximal increase in beating rate differs for each drug. Isoprenaline, soterenol, zinterol and formoterol rapidly enhanced the frequency while fenoterol, salmefamol and salbutamol took longer to produce their maximal effect. In contrast, the increase in beating rate induced by formoterol is very rapid but the decrease is also faster than for the other drugs. A comparison of the chemical structures of this limited series of compounds did not reveal any particular physico-chemical parameter which might account for their differential kinetics.

The variation in beating rate as a function of the β-agonist concentration did not, except for isoprenaline, give the classical regular sigmoïdal dose-response curves. This might indicate that the mechanism of action of the β_2-agonists is not unique and that a non-adrenergic mechanism could also be involved. However, for the different β-agonists, the intrinsic activity as well as the concentration at which the maximal effect occurred, revealed a close relationship between the response on heart cell and guinea-pig atria but not with trachea. This suggests that in the cultured rat heart cell, the frequency-response of the β_2-selective agonists is mediated mainly through β_1-adrenoceptors.

The fact that the molecules tested enhance the intracellular cyclic AMP content agrees with the work of Hazeki & Ui (1980). They showed that among other drugs, the β_2-selective agonists salbutamol, procaterol and trimetoquinol produced a cyclic AMP accumulation in dispersed heart cells. Since binding studies have revealed the presence in the atria of both β_1 and β_2-adrenoceptors, the existence of these two receptor subtypes in heart cell culture is also likely. It was thus necessary to determine whether cyclic AMP production in rat heart cells was mediated through β_1 or β_2-adrenoceptors. Comparison of the cyclic AMP accumulation induced by the β_2-agonists with their effects on guinea-pig trachea and atria, indicates a close relationship only with the latter. So, the increase in cyclic AMP content in cultured heart cells also appears to be mediated by the β_1-adrenoceptors.

Although β_2-agonists seem to act on heart cell cultures by a mechanism involving β_1-adrenoceptors, a comparison of the two responses shows that frequency enhancement already occurs at concentrations which have very little effect on cyclic AMP levels. These results contrast with the finding of Wollenberger & Irmler (1978) who for (−)-adrenaline observed similar dose-response curves for these two effects on cell culture grown on a rocker apparatus. Conversely, other authors have also reported a shift to the left of the chronotropic effect of isoprenaline compared to that of cyclic AMP levels in rat atria (Birnbaum, Abel, Amidon & Buckner 1975). One should stress here that the shift, obvious for isoprenaline, is only apparent for the other compounds at concentrations lower than 10^{-7}M. In any case our study confirms that substantial variations in beating rate can be observed while the changes in cyclic AMP levels remain low.

The relative parallelism between the frequency increases and cyclic AMP accumulation in our series of β_2-agonists could have been taken as further evidence that cyclic AMP is involved in the positive chronotropic action of β-agonists. In fact, one should be careful in drawing any definite conclusion since the link between the β_2-agonists and the increase in cyclic AMP formation is not clear. Thus, on rat ventricle homogenates, Minneman *et al.* (1979)

found that only fenoterol stimulated adenylate cyclase, whereas soterenol, salbutamol, salmefol and zinterol antagonized the isoprenaline-induced adenylate cyclase stimulation. One might argue that the differences in the effects of the β-agonists are due to the use of living cells as compared with broken membranes (Porzig, 1982). However, this does not seem to be the explanation since Buckner *et al.* (1978) and Hedberg *et al.* (1982) reported that soterenol and terbutaline produced no significant increase in cyclic AMP above baseline values on isolated atria (whereas isoprenaline did). An alternative explanation might be that the sensitivity of the β-adrenoceptor-adenylate cyclase system is magnified in the cultured heart cell, resulting in a higher cyclic AMP response for a given agonist concentration. Whatever the reason for such a discrepancy between heart cells and the whole atria or heart homogenate is, our results clearly establish that the β_2-selective agonists tested increase the intracellular cyclic AMP content and raise the question of the physiological relevance of the different models.

This study also shows that β_2-agonists, even those with high β_2-selectivity, are still relatively potent in eliciting positive chronotropic actions. In our heart cell model, a 15 to 30% increase in frequency is noted with the various compounds at a concentration of 10^{-8}M. Such effects which are also observed *in vivo*, limit the use of the β_2-agonists in asthma and for gynaecological purposes. Hence, β-agonists with higher selectivity for β_2-adrenoceptors have still to be discovered for therapeutic use.

In summary, in rat heart cell culture, the β_2-selective agonists induce an increase in beating rate. These effects appear to correspond with the β_1-adrenoceptor potency of the drugs on guinea-pig atria. Similarly, the β_2-agonists produce an accumulation of cyclic AMP in the cells, which is clearly connected to the β_1-activity of the molecules. This latter effect is at variance with previous work showing the inability of some β_2-agonists either to increase cyclic AMP levels on whole atria or on rat ventricle homogenate.

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