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EVIDENCE AGAINST β-ADRENOCEPTOR BLOCKING ACTIVITY OF DILTIAZEM, A DRUG WITH CALCIUM ANTAGONIST PROPERTIES

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1 In isolated spontaneously beating atria of the rat, diltiazem (0.01 to 0.1 μM) shifted the atrial rate concentration-response curves to isoprenaline to the right in a non-parallel manner and depressed their maxima. Under the same experimental conditions, (±)-propranolol (0.03 to 0.1 μM) behaved as a competitive β-adrenoceptor antagonist.

2 Whereas (±)-propranolol (IC_{50} = 12 nM) and isoprenaline (IC_{50} = 0.9 μM) inhibited (−)-[^{3}H]-dihydroalprenolol binding to rat brain membrane preparations, diltiazem failed to do so in concentrations up to 10 μM.

3 Diltiazem but not (±)-propranolol, antagonized the positive chronotropic responses to calcium in spontaneously beating rat atria.

4 It is proposed that diltiazem inhibited the tachycardia induced by isoprenaline through an effect on calcium which may be an essential modulator of the sequence of events linking the β-adrenoceptor activation and heart rate response.

Introduction

Diltiazem is a benzothiazepine derivative producing coronary vasodilatation in guinea-pig isolated hearts (Nagao, Sato, Nakajima & Kiyomoto, 1972) and in the intact dog (Sato, Nagao, Yamaguchi, Nakajima & Kiyomoto, 1971; Cavero, Boudot, Lefèvre-Borg & Roach, 1979). This property of diltiazem clearly distinguishes it from propranolol (Nayler, McInnes, Swann, Carson & Lowe, 1967). However, diltiazem was found to inhibit the positive chronotropic responses to isoprenaline in guinea-pig atria (Yamada, Shimamura & Nakajima, 1973). This antagonism is qualitatively different from that of (±)-propranolol since it was accompanied by a depression of the maximum of the concentration-response curve, which would be compatible with a non-competitive blockade of β-adrenoceptors. However, since diltiazem interferes with the transmembrane calcium influx in guinea-pig cardiac muscle (Nakajima, Hoshiyama, Yamashita & Kiyomoto, 1976), as well as rat taenia coli and myometrium (Magaribuchi, Nakajima & Kiyomoto, 1977a; Magaribuchi, Nakajima, Takenaga & Kiyomoto, 1977b), it is possible that the inhibition of isoprenaline tachycardia by this compound is due to its effects on calcium.

The present experiments were designed to investigate whether diltiazem inhibited the binding of (−)-[^{3}H]-dihydroalprenolol to β-adrenoceptors of rat membrane preparations and whether it interfered with the positive chronotropic responses to isoprenaline and calcium ions in spontaneously beating rat atria.

Methods

Male rats (C. River, Sprague Dawley) weighing 200 to 300 g were killed by cervical dislocation. The whole heart was immediately removed from the thoracic cavity and immersed in oxygenated (95% O_2 plus 5% CO_2) salt solution of the following composition (mM): NaCl 120, KCl 5.6, $CaCl_2$ 2.2, $MgCl_2$ 2.1, $NaHCO_3$ 25.0 and glucose 10.0. The atria were dissected free of all extraneous tissues, placed in a 30 ml organ bath thermostatically controlled at 31°C and subjected to a resting tension of approximately 0.5 g. After a 60 min equilibration period the experimental procedure was started.

Concentration-response curves to isoprenaline

Concentration-response curves to isoprenaline were determined before and 30 min after the exposure of

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the atria to (±)-propranolol (0.03 and 0.1 μM), diltiazem (0.01 and 0.1 μM) or their solvent (saline: 0.1 ml). The time between the first control and the second concentration-response curve was approximately 90 min.

The atrial rate response to isoprenaline is expressed as a percentage of the maximal response to a supramaximal concentration of isoprenaline. Since diltiazem and (±)-propranolol slightly decreased (10 to 20%) the intrinsic atrial rate, the maximal response to isoprenaline after these treatments was taken as the difference between the baseline atrial rate after 30 min exposure to these compounds and the maximal response to isoprenaline during the corresponding control concentration-response curve. Under our experimental conditions, when a competitive β-adrenoceptor antagonist decreased resting atrial rate, the maximal increase in rate obtained after a supramaximal concentration of isoprenaline was generally the same as that observed under control conditions.

The pD_2, pA_2 and pD'_2 values were calculated as described by Van Rossum (1963).

Atrial rate responses to calcium chloride

In a number of rat atria, an attempt was made to determine atrial rate concentration-response curves to calcium chloride. However, the reproducibility of these curves was poor, both within and between preparations. It was, therefore, decided to study the effects of diltiazem (0.1 μM), (±)-propranolol (0.1 μM) or their solvent (saline) on the tachycardia induced by a single concentration of calcium chloride (2.2 mM). Only atrial preparations giving maximal atrial rate increases over 40 beats/min were used for these experiments (about 67%). Each treatment was added to the organ bath 10 min after calcium chloride (at which time the tachycardia produced by calcium chloride reached a steady state) and its effect was followed for the next 30 min. A matched series of experiments was carried out in atria in which calcium was substituted with saline (0.1 ml).

Binding of (−)-[^{3}H]-dihydroalprenolol

The binding of (−)-[^{3}H]-dihydroalprenolol ([^{3}H]-DHA) to rat brain membranes was performed as described by Alexander, Davis & Lefkowitz (1975). Specific binding was defined as that portion of total [^{3}H]-DHA binding inhibited in the presence of 10 μM (±)-propranolol and ranged from 50 to 75% of the total binding. The ability of a compound to inhibit the binding of [^{3}H]-DHA (10 nM) was expressed in terms of its IC_{50} value which is the concentration of a drug required to reduce the specific binding of [^{3}H]-DHA by 50%.

Analysis of results

Results are given as means ± s.e. mean. A paired t test was used to evaluate the significance of responses within the same group. The slopes of the concentration-response sigmoid curves were calculated by using a logistic function model (Gomeni & Gomeni, 1978). A two-way analysis of variance (ANOVA) or a t test was used to assess significant differences.

Drugs

The following drugs were used: (−)-[^{3}H]-dihydroalprenolol (specific activity: 51.1 Ci/mmol; N.EN. Chemicals, GmbzH), diltiazem hydrochloride (Tanabe Seiyaku), (±)-isoprenaline sulphate (Labaz) and (±)-propranolol hydrochloride (ICI).

Results

Effects of diltiazem and (±)-propranolol on concentration-response curves to isoprenaline

The spontaneous beating rate of 23 isolated rat atria in which isoprenaline concentration-response curves were studied was 207 ± 4 beats/min. Addition of isoprenaline to this preparation increased atrial rate in a concentration-related manner, the maximal response to the highest concentration of isoprenaline being 84 ± 3 beats/min (Figure 1).

A 30 min exposure of atria (n = 3) to the solvent (saline) of diltiazem or (±)-propranolol caused no significant change in atrial chronotropism (−4%), the slope or the maximum of the isoprenaline concentration-response curve. The pD_2 values of isoprenaline before and after 30 min exposure to the solvent were 8.98 ± 0.1 (n = 3) and 8.75 ± 0.14, respectively.

Atrial rates were decreased by 8 and 20 or 17 and 22% after 30 min exposure to 0.01 and 0.1 μM diltiazem or 0.03 and 0.1 μM (±)-propranolol, respectively. However, in the case of (±)-propranolol, despite this reduced resting atrial rate, the peak rate attained with a supramaximal concentration of isoprenaline was not different (284 ± 7 beats/min, n = 6) from that obtained (293 ± 11 beats/min) under control conditions.

(±)-Propranolol displaced to the right in a parallel manner the control atrial rate concentration-response curves to isoprenaline (Figure 1). Its pA_2 value was calculated as 8.69 ± 0.14 (n = 6), from results with the two concentrations of (±)-propranolol. In contrast, diltiazem produced a concentration-dependent shift to the right of the isoprenaline concentration-response curve which was not parallel and was ac-

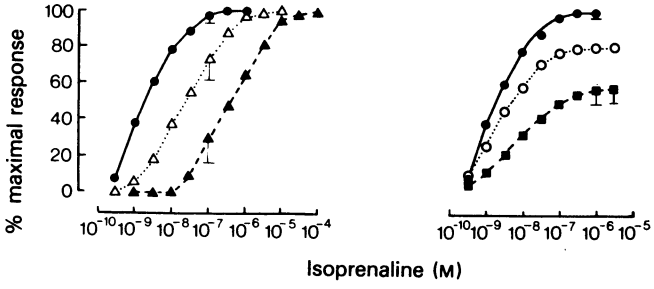

Figure 1 Concentration-effect curves to isoprenaline (as % of maximal response: see Methods) in spontaneously beating rat atria before (●; control: $n = 20$) and 30 min after exposure to (±)-propranolol (0.03 øM: △; 0.1 μM: ▲; $n = 3$/group) and diltiazem (0.01 μM: ○; 0.1 μM: ■; $n = 7$/group). The slopes and the maxima of the sigmoid curves to isoprenaline obtained before and after (±)-propranolol are similar. The two concentration of diltiazem depressed significantly ($P < 0.05$: t test) the maxima of the curves to isoprenaline. However, only the slope of the curve after the highest concentration of diltiazem was significantly flatter than that of the control ($P < 0.005$: t test).

companied by a significant decrease in maximal response (Figure 1). Its pD'_2 value against isoprenaline was found to be 7.09 ± 0.10 ($n = 14$) which was calculated from the data obtained with the two concentrations of diltiazem (Figure 1).

Effects of diltiazem and (±)-propranolol on the positive chronotropic effects of calcium

Addition of calcium (2.2 mM) to spontaneously beating rat atria (mean baseline rate 184 ± 4 beats/min:

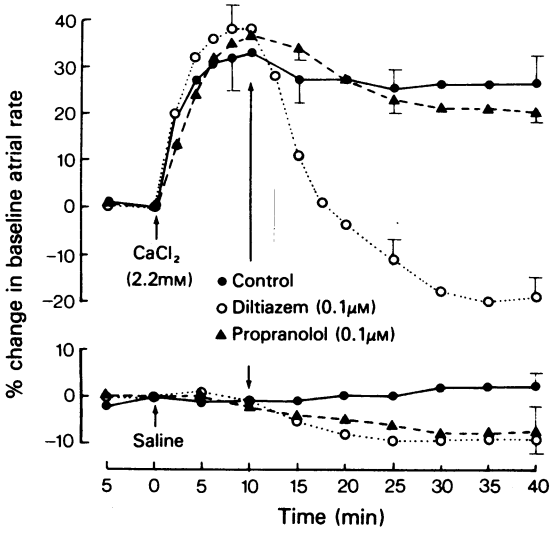

Figure 2 Antagonism by diltiazem of the positive chronotropic effects induced by calcium chloride. Results are expressed as % of control baseline atrial rate. Shown are the effects of diltiazem (0.1 μM: ○; $n = 6$), (±)-propranolol (0.1 μM: ▲; $n = 6$) or their solvent (saline: ●; $n = 8$) on the tachycardia induced by calcium chloride (2.2 mM) in spontaneously beating rat atria. The effects of the three treatments on the basal rate ($n = 4$–5/group) are also shown. There was no difference in the effects produced by (±)-propranolol and saline on the calcium-induced tachycardia whereas the effects of diltiazem were significantly different from those of (±)-propranolol and saline (ANOVA on the changes observed at 7, 15 and 30 min after treatment). The basal atrial rate effects of diltiazem and (±)-propranolol were significantly different from those of saline ($P < 0.05$: ANOVA).

$n = 18$) bathed in the standard salt solution increased heart rate by 65 ± 7 beats/min ($n = 20$).

(±)-Propranolol decreased this tachycardia only slightly more than saline (control) and, this effect was of the same magnitude as that observed with (±)-propranolol in atria in which no calcium had been added (Figure 2). In contrast, diltiazem antagonized the positive chronotropic effect of calcium and decreased atrial rate to values lower than those present before the addition of calcium (Figure 2) and similar to those produced by this compound in rat atria not exposed to calcium (Figure 2).

Effects of diltiazem, isoprenaline and (±)-propranolol on [^{3}H]-dihydroalprenolol binding

Isoprenaline and (±)-propranolol inhibited [^{3}H]-DHA binding to rat brain membrane preparations. Their IC_{50} values were 0.9 μM and 12 nM, respectively. Diltiazem at concentrations up to 10 μM failed to inhibit the [^{3}H]-DHA binding.

Discussion

Diltiazem antagonized the chronotropic responses to isoprenaline in spontaneously beating rat atria, but, this effect was entirely different from that observed with (±)-propranolol since it was characterized by a depression of the response to a supramaximal concentration of isoprenaline. The question of whether or not diltiazem reduced the maximum through an irreversible occupation of β-adrenoceptors is difficult to clarify on the basis of functional studies in rat atria. We, therefore, turned to a radioligand technique using [^{3}H]-DHA which has been demonstrated to bind to β-adrenoceptors in a variety of tissues (Williams & Lefkowitz, 1978). In contrast to diltiazem, both the β-adrenoceptor agonist, isoprenaline and the β-adrenoceptor antagonist, propranolol, were found to inhibit the binding of [^{3}H]-DHA to rat brain membrane preparations. When this study was terminated, it was reported that diltiazem does not bind to myocardial β-adrenoceptors (Flaim, Tarka, Flaim & Zelis, 1979). Therefore, the apparent non-competitive type of antagonism produced by diltiazem against the chronotropic responses to isoprenaline was probably not due to an action at the level of the β-adrenoceptor.

Diltiazem, in contrast to (±)-propranolol, antagonized the tachycardia induced by calcium. Since the sequence of events between drug-receptor binding and the final biological response is a poorly understood phenomenon, it is conceivable that two drugs inhibiting the effects of the same agonist may do so either by competing for the same receptors or by affecting one link of the subsequent chain of events. If the isoprenaline-induced activation of β-adrenoceptors is a calcium-dependent process and this pool of calcium is affected by diltiazem, then the inhibition of isoprenaline tachycardia by diltiazem is due to an impaired calcium availability for the β-adrenoceptor activation-effector coupling system. The results of Toda (1969) in which the change in sensitivity of the S–A node to noradrenaline varied directly with the calcium concentration in the bathing medium, indicate that calcium may be a modulator of the chronotropic effects due to β-adrenoceptor activation. It is, therefore, possible that drugs like diltiazem which interfere with transmembrane calcium influx (Nakajima *et al.*, 1976; Migaribuchi *et al.*, 1977a, b) or calcium availability for certain cellular actions can reduce the biological responses to agonists requiring this ion for the excitation-contraction coupling. This suggestion is supported by the observation that diltiazem antagonized in a non-competitive manner the positive chronotropic responses to histamine (unpublished personal observations) in guinea-pig atria and Levi & Pappano (1978) have suggested that calcium may act as modulator of this effect of histamine. Furthermore, several reports indicate that verapamil, a well known inhibitor of the calcium-mediated slow inward current, antagonizes cardiac responses to β-adrenoceptor agonists and this effect is not due to an action on β-adrenoceptors, but occurs somewhere in the chain of events triggered by the activation of the β-adrenoceptors and leading to the mechanical response (Nayler, McInnes, Swann, Price, Carson, Race & Lowe, 1968; Watanabe & Besh, 1974; Bristow & Green, 1977).

It is concluded that the inhibition of responses to β-adrenoceptor agonists by calcium antagonists, such as diltiazem and verapamil, is probably the direct consequence of their effects on calcium availability for cellular processes.

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References

ALEXANDER, R.W., DAVIS, J.N. & LEFKOWITZ, R.J. (1975). Direct identification and characterisation of β-adrenergic receptors in rat brain. *Nature*, **258**, 437–440.

BRISTOW, M.R. & GREEN, R.D. (1977). Effect of diazoxide, verapamil, and compound D600 and isoproterenol and calcium-mediated dose-response relationships in isolated rabbit atrium. *Eur. J. Pharmac.*, **45**, 267–279.

CAVERO, I., BOUDOT, J.P., LEFÈVRE-BORG, F. & ROACH A.G. (1979). Pharmacological evaluation of diltiazem and its deacetylated metabolite in several animal species. In

New Therapy with a Calcium Antagonist, Diltiazem. Hakone Symposium 178. ed. Bing, R.J. pp. 61–88. Amsterdam-Princeton: Excerpta Medica/Elsevier.

FLAIM, S.F., TARKA, L.A., FLAIM, K.E. & ZELIS, R. (1979). Binding capacity of diltiazem to myocardial β-adrenergic receptors. *Clinical Res.*, **27**, 165A (Abs).

GOMENI, R. & GOMENI, C. (1978). Interactive graphic program for automatic analysis of dose-response curves. Abs. 996, *7th International Congress Pharmacology*, Paris.

LEVI, R. & PAPPANO, A.J. (1978). Modification of the effects of histamine and norepinephrine on the sinoatrial node pacemaker by potassium and calcium. *J. Pharmac. exp. Ther.*, **204**, 625–633.

MAGARIBUCHI, T., NAKAJIMA, H. & KIYOMOTO (1977a). Effects of diltiazem and lanthanum ions on the potassium contracture of isolated guinea-pig smooth muscle. *Jap. J. Pharmac.*, **27**, 333–339.

MAGARIBUCHI, T., NAKAJIMA, H., TAKENAGA, H. & KIYOMOTO, A. (1977b). Effect of diltiazem on the transmembrane potential of isolated guinea-pig taenia coli. *Jap. J. Pharmac.*, **27**, 319–322.

NAGAO, T., SATO, M., NAKAJIMA, H. & KIYOMOTO, A. (1972). Studies on a new 1,5-benzothiazepine derivative (CRD-401)—II. Vasodilator action. *Jap. J. Pharmac.*, **22**, 1–10.

NAKAJIMA, H., HOSHIYAMA, M., YAMASHITA, K. & KIYOMOTO, A. (1976). Electrical and mechanical responses to diltiazem in potassium depolarized myocardium of the guinea-pig. *Jap. J. Pharmac.*, **26**, 571–580.

NAYLER, W.G., MCINNES, I., SWANN, J.B., CARSON, V. & LOWE, T. (1967). Effect of propranolol, a beta-adrenergic antagonist, on blood flow in the coronary and other vascular fields. *Am. Heart J.*, **73**, 207–216.

NAYLER, W.G., MCINNES, I., SWANN, J.B., PRICE, V., CARSON, V., RACE, D. & LOWE, T. (1968). Some effects of iproveratril (isoptin) on the cardiovascular system. *J. Pharmac. exp. Ther.*, **161**, 247–261.

SATO, M., NAGAO, T., YAMAGUCHI, I., NAKAJIMA, H. & KIYOMOTO, A. (1971) Pharmacological studies on a new 1,5-benzothiazepine derivative (CRD-401)—I. Cardiovascular actions. *Arzneim. Forsch.*, **21**, 1338–1343.

TODA, N. (1969). Effects of calcium, sodium and potassium ions on contractility of isolated atria and their responses to noradrenaline. *Br. J. Pharmac.*, **36**, 350–367.

VAN ROSSUM, J.M. (1963). Cumulative dose-response curves—II. Techniques for making of dose-response curves in isolated organs and the evaluation of drug parameters. *Archs Int. Pharmacodyn.*, **143**, 299–330.

WATANABE, A.M. & BESCH, H.R. (1974). Cyclic adenosin monophosphate modulation of slow calcium influx channels in guinea-pig heart. *Circulation Res.*, **35**, 316–324.

WILLIAMS, L.T. & LEFKOWITZ, R.J. (1978). *Receptor Binding Studies in Adrenergic Pharmacology.* pp. 83–109. New York: Raven Press.

YAMADA, K., SHIMAMURA, T. & NAKAJIMA, H. (1973). Studies on a new 1,5-benzothiazepine derivative (CRD-401)—V. Antiarrhythmic actions. *Jap. J. Pharmac.*, **23**, 321–328.

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