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In vivo effect of methyl-quinuclidinyl-benzylate on myocardial β-adrenoceptor density

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Abstract

The muscarinic receptor antagonist methyl-quinuclidinyl-benzylate decreased myocardial β-adrenoceptor density B_{max}: 20.4 ± 2.4 pmol/ml tissue versus 33.3 ± 4 pmol/ml tissue in control dogs ($P < 0.001$), as assessed by using [^{11}C]CGP-12177 (((2*S*)-4-(3-*t*-butyl-amino-2 hydroxypropoxy)-benzimidazol-2-one)) and positron emission tomography. In contrast, atropine did not induce any change in B_{max}: 33.7 ± 3.6 pmol/ml tissue. We hypothetized that methyl-quinuclidinyl-benzylate induced the release of norepinephrine from sympathetic nerve terminals, an effect which could be blocked by guanethidine. Guanethidine alone (10 mg/kg) did not change B_{max}: 35.5 ± 6 pmol/ml tissue. Guanethidine + methyl-quinuclidinyl-benzylate did not induce any significant change in B_{max}: 31.5 ± 5.1 pmol/ml tissue. Therefore, it seems likely that methyl-quinuclidinyl-benzylate acts at the presynaptic level, probably inducing the release of norepinephrine which then causes a down-regulation of β-adrenoceptors.

Keywords: Atropine; Methyl-quinuclidinyl-benzylate; CGP-12177; PET (positron emission tomography); Heart; β-Adrenoceptor; Muscarinic receptor

1. Introduction

The chronotropic function of atrial muscarinic acetylcholine receptors is well known. Ventricular muscarinic acetylcholine receptors are scarce compared to the atrial receptors (Nathanson, 1987) and their physiological functions remain unclear. Stimulation of muscarinic acetylcholine receptors inhibits adenylate cyclase activity (Nathanson, 1987), but, acetylcholine, intracoronary injected, has no – or very little – effect on ventricular inotropism (Landzberg et al., 1994). Most ventricular muscarinic receptors are located on myocytes but some are located on sympathetic nerve endings (Sharma and Banerjee, 1978). The later regulate norepinephrine release (Lindmar et al., 1968). The impact of muscarinic agonist and antagonist administration on norepinephrine turnover has been extensively studied (Löffelholz and Muscholl, 1969; Starke, 1977; Muscholl, 1980; Vanhoutte and Levy, 1980). However, the impact on β-adrenoceptor density has not been studied. To assess the density of muscarinic acetylcholine receptors in vivo, we used as positron emission tomography (PET) ligand the muscarinic receptor antagonist [^{11}C]methyl-quinuclidinyl-benzylate (Mazière et al., 1981; Syrota et al., 1985). We observed that dogs given methyl-quinuclidinyl-benzylate always had a significantly lower density of myocardial β-adrenoceptors. Therefore, this unexpected effect of methyl-quinuclidinyl-benzylate on β-adrenoceptor density was in vivo compared to that of atropine using PET and the β_1-β_2-adrenoceptor antagonist [^{11}C]CGP-12177 (((2*S*)-4-(3-*t*-butylamino-2 hydroxypropoxy)-benzimidazol-2-one); Hertel et al., 1983; Staehelin and Hertel, 1983). The possible mechanism of action of methyl-quinuclidinyl-benzylate on β-adrenoceptor density was also investigated. Furthermore, the functional aspect of changes in β-adrenoceptor density was assessed by studying the changes in the left ventricular inotropic response to the β_1-β_2-adrenoceptor agonist dobutamine.

2. Materials and methods

Female beagle dogs (mean weight 11 kg) were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

Six dogs were used as controls for the determination of left ventricular β-adrenoceptor density. Five dogs were

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pretreated with methyl-quinuclidinyl-benzylate 750 μg i.v. 2 h prior to the PET experiment. This dose, which saturates more than 95% of the myocardial binding sites in beagle dogs of this size, was similar to that used for the PET determination of muscarinic acetylcholine receptor density (Delforge et al., 1990).

To elucidate the possible effect of methyl-quinuclidinyl-benzylate on myocardial catecholamine release, the same five dogs were pretreated with guanethidine 2 weeks later. Guanethidine (10 mg/kg i.v.; Gaffney et al., 1962) was administered on each of the 2 days prior to the PET study. To verify the blocking action of guanethidine on the release of norepinephrine, dogs were i.v. injected with tyramine (100 μg/kg over 60 s) at the end of the PET study with continuous monitoring of intra-arterial pressure (femoral catheter) and heart rate. Tyramine did not induce significant changes (Deitchman et al., 1980).

Two to four weeks later, the same five dogs were pretreated with both guanethidine and methyl-quinuclidinyl-benzylate (as above) and underwent the PET measurement of β-adrenoceptor density. At the end of the PET study, the same tyramine test was performed; tyramine did not induce any significant change.

Five other dogs were pretreated with atropine (500 μg i.v. four times, 30 min apart, the first dose being administered 2 h before the PET experiment). This dose was chosen because, in pentobarbital anaesthetized dogs, the adequacy and duration of muscarinic blockade was similar to that elicited by methyl-quinuclidinyl-benzylate (750 μg), as shown by the absence of tachycardia and hypotension following acetylcholine 10 μg/kg i.v. (Vatner et al., 1979).

In order to assess the functional changes induced by methyl-quinuclidinyl-benzylate or atropine, the same two groups of dogs underwent two gated blood pool studies (control and the muscarinic receptor antagonist in random order), 1–2 weeks apart. The changes in response of left ventricular function to dobutamine infusion were measured.

2.1. PET studies

The pharmacologically active enantiomer (2*S*) CGP-12177, ((2*S*)-4-(3-*t*-butylamino-2 hydroxypropoxy)-benzimidazol-2-one) was synthetized and labeled with ^{11}C as previously described (Hammadi and Crouzel, 1991). The enantiomeric excess was greater than 98%. [^{11}C]CGP-12177, in the S form, was obtained with a specific radioactivity ranging from 350 to 1200 mCi/μmol.

2.1.1. PET experimental protocol

Dogs were anesthetized with pentobarbital, intubated and artificially ventilated. They were positioned in the TTV01 time-of-flight PET scanner (LETI, CEA, Grenoble, France). Each slice was 13 mm thick and spatial transverse resolution was 12 mm. Transmission scans were obtained with a rotating ^{68}Germanium source and used for attenuation correction of the emission scans.

The graphical method used for the determination of β-adrenoceptor density (Delforge et al., 1991) is based on a specific experimental protocol related to the kinetics of CGP-12177. The receptor concentration was estimated by using two experimental myocardial concentration values calculated from the PET time-concentration curve. The protocol included two i.v. injections: a trace dose of [^{11}C]CGP-12177 (3–5 nmol) at the beginning of the experiment and, 30 min later, a mixture of labeled (6–8 nmol) and unlabeled (35 nmol) CGP-12177. The PET examination lasted 70 min. The dynamic series consisted of 66 frames (18 × 10 s, 7 × 1 min, 5 × 2 min, 2 × 5 min, 18 × 10 s, 7 × 1 min, 5 × 2 min, 4 × 5 min images).

2.1.2. PET data processing

The myocardial time-concentration curve was measured from a region of interest encompassing the left ventricular myocardium. [^{11}C]CGP-12177 concentrations were obtained after correction for ^{11}C decay and expressed as pmol/g after normalization by using the specific radioactivity measured at the beginning of the PET experiment. Data were corrected for a partial volume effect by using post-mortem measurements of left ventricular wall thickness (four dogs) and a recovery factor measured on a heart phantom. The ratio of true-to-measured concentrations was equal to 0.45 for a thickness of 12 mm in the phantom calibration experiment performed on the TTV01 PET system (12 mm is the mean myocardial thickness of a beagle dog weighing 10–11 kg). Therefore, true concentrations were obtained by dividing the measured concentration values by this 0.45 coefficient.

2.1.3. Calculation of β-adrenoceptor density

The receptor concentration was estimated by using two experimental myocardial concentration values calculated from the PET time-concentration curve (Delforge et al., 1991). This approach relies on differences in the radiotracer kinetics when radiotracer injected alone or with an excess of unlabeled ligand. It is based on the following differential equation:

$$dB(t) = (k_{+1}/V_R)(B_{max} - B(t))F(t) - k_{-1}B(t)$$

where $B(t)$ and $F(t)$ are the molar concentrations of bound and free ligand, respectively; V_R is the volume of reaction for free ligand in tissue; k_{+1} is the association rate constant; k_{-1} is the dissociation rate constant. The method is based on an uptake measurement where association of the tracer to the receptor dominates the kinetics and the small effect of dissociation ($k_{-1}B(t)$ in the equation) is accounted for in the analysis by exponential extrapolation.

2.2. In vitro determination of β-adrenoceptor density

β-Adrenoceptor density was measured in three control dogs and three dogs pretreated with methyl-quinuclidinyl-

benzylate (750 μg i.v.) injected 2 h before the animals were killed with an overdose of pentobarbital. The heart was rapidly removed and left ventricular samples (2–4 g) were stored in liquid nitrogen. [^{3}H]CGP-12177 (purchased from New England Nuclear; specific activity: 54 Ci/mmol) was used. Membrane preparation was performed as previously described (Merlet et al., 1993). Membrane homogenates (300 μl) were prepared to a final volume of 2 ml with buffer (50 mM Tris, 10 mM $MgCl_2$, pH 7.4). Increasing concentrations of [^{3}H]CGP-12177 (from 0.2 to 3 nM) were added. The incubation conditions (37°C for 60 min) ensured that equilibrium was reached between the receptors and the radioligand. The reaction was terminated by rapid vacuum filtration through Whatman GF/C filters. Filters were washed with a 15 ml excess of ice-cold buffer. These filters were then dried and placed into 5 ml scintillation fluid (Insta-Gel, Packard). A liquid scintillation counter (Packard SL 2000, 80% efficiency) was used to determine the sample radioactivity. Non-specific binding was detected in the presence of 3 μM of propranolol and averaged 10–15% of total binding. Maximum density and apparent affinity (K_d) were assessed in each individual experiment using a non-linear least square regression program. Protein content was measured according to Lowry et al. (1951). The mean value was 92 ± 4 mg/g tissue.

2.3. Gated blood pool studies

Gated blood pool studies (1–2 weeks apart) were performed, in a random order, in basal conditions and after pretreatment with methyl-quinuclidinyl-benzylate (750 μg i.v., 2 h before the gated blood pool study) or atropine (500 μg i.v. four times, 30 min apart). Dogs were anesthetized and artificially ventilated as during the PET experiments. Red blood cells were in vivo labeled with 25–30 mCi Tc 99m. During each experiment, left ventricular function was assessed before and during dobutamine infusion at incremental doses of 2, 4 and 6 μg/kg/min. These doses were chosen to avoid marked changes in heart rate and blood pressure (Valette et al., 1992). Scintigraphic acquisition was started 3 min after the beginning of dobutamine infusion, thereby ensuring the stability of the hemodynamic parameters (heart rate and femoral artery pressure were continuously monitored). Gated acquisition lasted 4–6 min in order to obtain 400 kcounts per frame (16 frames per cardiac cycle, matrix format 64 × 64, pixel size = 4.5 mm). Scintigraphic data were processed with standard software (Standke et al., 1983). The measured parameters included heart rate, mean blood pressure, left ventricular ejection fraction.

2.4. Plasma norepinephrine determination

At the beginning of each PET study, venous blood samples were drawn. Plasma norepinephrine concentrations were determined by high-pressure liquid chromatography (Bove et al., 1984).

2.5. Statistical analysis

All parameters are expressed as the mean value ± standard deviation. The different β-adrenoceptor densities obtained in the five conditions (baseline, pretreatment with methyl-quinuclidinyl-benzylate, atropine, guanethidine alone, both guanethidine and methyl-quinuclidinyl-benzylate) were compared by using an analysis of variance and Bonferroni's test. Parameters measured during gated blood pool studies were compared by using an analysis of variance for repeated measures. A $P < 0.05$ was considered statistically significant.

3. Results

3.1. PET studies

Left ventricular β-adrenoceptor density was 33.3 ± 4 pmol/ml tissue in control dogs (Fig. 1). Following pretreatment with methyl-quinuclidinyl-benzylate, there was a significant decrease in B_{max} = 20.4 ± 2.4 pmol/ml tissue ($P < 0.001$). In contrast, pretreatment with atropine did not induce any change in B_{max} = 33.7 ± 3.6 pmol/ml tissue ($P = 0.2$). Pretreatment with guanethidine alone or with guanethidine + methyl-quinuclidinyl-benzylate did not induce any significant change in B_{max} = 35.5 ± 6 pmol/ml tissue ($P = 0.2$) and B_{max} = 31.5 ± 5.1 pmol/ml tissue ($P = 0.1$), respectively. Plasma norepinephrine concentrations were similar in control, methyl-quinuclidinyl-benzylate-pretreated, atropine-pretreated, guanethidine-pretreated and guanethidine + methyl-quinuclidinyl-benzylate-pretreated dogs (251 ± 35 pg/ml, 232 ± 38 pg/ml, 258 ± 32 pg/ml, 264 ± 42 pg/ml and 248 ± 46 pg/ml, respectively, $P > 0.1$).

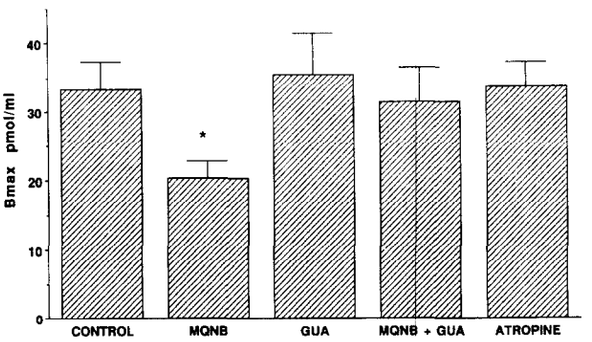

Fig. 1. Changes in β-adrenoceptor densities in control dogs, in dogs pretreated with methyl-quinuclidinyl-benzylate (MQNB), atropine, guanethidine (GUA) and methyl-quinuclidinyl-benzylate + guanethidine (mean values ± S.D., $n = 5$ per group, except $n = 6$ for control group). * A significant difference versus control values.

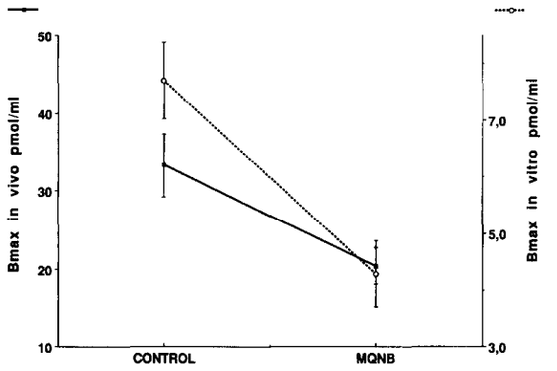

Fig. 2. Comparison of changes in β-adrenoceptor densities measured in vivo and in vitro in control dogs and in dogs pretreated with methyl-quinuclidinyl-benzylate (MQNB; mean values ± S.D., in vivo $n = 5$ per group, in vitro $n = 3$ per group).

3.2. In vitro β-adrenoceptor density

A statistically significant decrease in β-adrenoceptor density (Fig. 2) following treatment with methyl-quinuclidinyl-benzylate was observed: B_{max} control = 91 ± 8 fmol/mg protein, B_{max} treatment = 51 ± 7 fmol/mg protein ($P < 0.001$). There was no change in affinity constant: K_d control = 2.3 ± 0.4 nM and K_d treatment = 1.76 ± 0.3 nM ($P = 0.1$).

3.3. Gated blood pool studies and response to dobutamine infusion

There was no statistically significant difference in baseline parameters (Figs. 3–4) between control, methyl-quinuclidinyl-benzylate- and atropine-pretreated dogs. The lack of a significant change in heart rate was mainly due to the large standard deviation observed in anesthetized dogs. In fact, in methyl-quinuclidinyl-benzylate- or atropine-pretreated dogs, the heart rate was most of the time higher than that measured under control conditions (155 ± 30, 153 ± 30 and 130 ± 34 beats/min before dobutamine infusion, respectively). Dobutamine, even at the highest dosage, did not induce any significant change in heart rate (atropine: 165 ± 35 beats/min; methyl-quinuclidinyl-benzylate: 166 ± 33 beats/min; control: 134 ± 31 beats/min). Blood pressure also remained unchanged in controls or following methyl-quinuclidinyl-benzylate (Fig. 3). A significant ($P = 0.006$) decrease in the responsiveness of left ventricular function (Fig. 4) was observed in dogs pretreated with methyl-quinuclidinyl-benzylate. In contrast, muscarinic blockade with atropine induced an increase in both left ventricular function ($P = 0.002$; Fig. 4) and blood pressure ($P = 0.01$; Fig. 3).

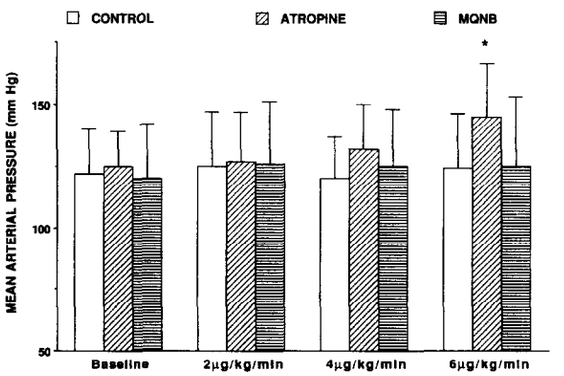

Fig. 3. Changes in mean arterial pressure (mean values ± S.D., $n = 5$ per group) during dobutamine infusion in control dogs, in dogs pretreated with methyl-quinuclidinyl-benzylate or atropine. * A significant difference between control dogs and dogs pretreated with methyl-quinuclidinyl-benzylate (MQNB).

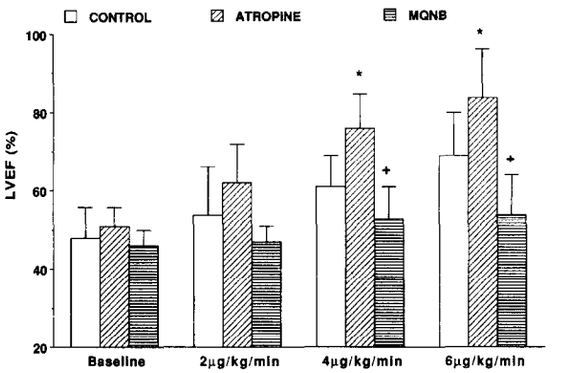

Fig. 4. Changes in left ventricular ejection fraction (LVEF, mean values ± S.D., $n = 5$ per group) during dobutamine infusion in control dogs, in dogs pretreated with methyl-quinuclidinyl-benzylate or atropine. * A significant difference between control dogs and dogs pretreated with atropine; + a significant difference between control or atropine pretreated dogs and methyl-quinuclidinyl-benzylate pretreated dogs.

4. Discussion

In pentobarbital anesthetized dogs, acute administration of atropine had no detectable effect on β-adrenoceptor density, but increased the responsiveness to a β-adrenoceptor agonist infusion, a result found by others (in dogs: Vatner et al., 1979; in humans: Landzberg et al., 1994).

Methyl-quinuclidinyl-benzylate induced a decrease in surface-bound β-adrenoceptors. The reduction of β-adrenoceptor density was in a similar range whether determined in vivo (reduction of 37%) or ex vivo (reduction of 46%). A possible reason for this reduction is the elevation in myocardial interstitial norepinephrine concentration (Delehanty et al., 1994). In support of this is the effect of guanethidine, which combines the adrenergic neuronal blocking action of bretylium with the depleting action of reserpine (Maxwell, 1980). The dosage of guanethidine and the timing of administration were selected – after several PET experiments – to avoid the reserpine-like action of guanethidine which could per se modify the number of β-adrenoceptors. The blocking action of guanethidine on norepinephrine release was confirmed in

all dogs by the lack of chronotropic response to tyramine infusion.

The PET method estimates the number of available β-adrenoceptors (and not the total number of receptors). It is unlikely that the decrease in β-adrenoceptor density is due to the occupancy of β-adrenoceptor sites by an excess of norepinephrine following methyl-quinuclidinyl-benzylate infusion, as the affinity of CGP-12177 for the receptor is greater than that of norepinephrine (Staehelin and Hertel, 1983). Furthermore, the absence of a significant increase in baseline left ventricular function after methyl-quinuclidinyl-benzylate administration makes this hypothesis unlikely. An effective change in the volume of reaction (V_r) for CGP-12177 following treatment with methyl-quiniclidinyl-benzylate cannot be ruled out. This hypothesis is unlikely since pretreatment with guanethdine + methyl-quiniclidinyl benzylate did not induce any change in B_{max}.

To confirm the physiological importance of the decreased number of β-adrenoceptors, the response to dobutamine was assessed. Radioligand binding and physiologic data suggest that dobutamine acts mainly on the β_1-adrenoceptor, but also interacts with α_1- and β_2-adrenoceptors (Deighton et al., 1992). However, dobutamine can induce load and heart rate changes that may interfere with the contractile response to the drug. In the present study, no significant changes of heart rate or blood pressure were noticed in control dogs. The changes in left ventricular ejection fraction do represent a coarse, but acceptable, method to assess in vivo β-adrenoceptor-mediated responsiveness. The contractile response to dobutamine infusion was markedly different after administration of both muscarinic receptor antagonists.

Methyl-quinuclidinyl-benzylate induced a rapid (< 120 min) 'internalization' of surface receptors. In fact, the loss of surface-bound β-adrenoceptors may be very rapid: a decrease by 50% after 10 min exposure to isoproterenol has been described (Limas and Limas, 1984). Following exposure to a β-adrenoceptor agonist, short-term desensitization is triggered by two kinases (β-adrenoreceptor kinase or c-AMP-dependent protein kinase A; Lohse, 1992). There are no data concerning a possible action of muscarinic receptor antagonists on these kinases. Therefore, we hypothetized that methyl-quinuclidinyl-benzylate was acting at the presynaptic level. Muscarinic acetylcholine receptors, although mainly located on myocytes, are also present on sympathetic nerve endings (Sharma and Banerjee, 1978). Both presynaptic muscarinic acetylcholine receptors and α_2 mechanisms contribute to regional variations in the rate constant of norepinephrine turnover (Schmid et al., 1986).

There is a discrepancy between our findings and those obtained with atropine by Levy and Blattberg (1976). The overflow of myocardial norepinephrine induced by electrical sympathetic stimulation was reduced by a high dose (1 mg/kg) of atropine that is supposed to block the muscarinic effects of acetylcholine and to competitively antagonize the action of acetylcholine on nicotinic receptors (Lindmar et al., 1968).

No change in ventricular norepinephrine turnover was found by Schmid et al. (1986) in conscious guinea-pigs treated with quinuclidinyl-benzylate while yohimbine or quinuclidinyl-benzylate + yohimbine increased norepinephrine turnover although the dose of muscarinic receptor antagonist was similar to that which we used (70–80 μg/kg). This discrepancy may be due to inter-species differences, cold stress used in guinea-pigs to induce a significant increase in the release of norepinephrine, or to the different pharmacological properties of the two muscarinic receptor antagonists. In fact, quinuclidinyl-benzylate is lipophilic (Gossuin et al., 1984) and crosses the blood-brain barrier (Varastet et al., 1992). Therefore, a central effect could modify norepinephrine turnover. Methyl-quinuclidinyl-benzylate is hydrophilic and does not cross the blood-brain barrier (Mazière et al., 1981). Gallamine, a selective M_2 muscarinic receptor antagonist with nicotinic properties (Stockton et al., 1983), does not induce desensitization of β-adrenergic receptors in myocytes free of innervation (Limas and Limas, 1984). In contrast, in a vein strip preparation, when norepinephrine release in response to nerve stimulation is first depressed by acetylcholine, gallamine increases (50% above control levels) norepinephrine overflow (Verbeuren and Vanhoutte, 1976). In isolated rabbit or guinea-pig hearts, atropine administered after acetylcholine leads to a 10-fold increase of norepinephrine release (Lindmar et al., 1968). Lindmar suggested that atropine blocked the mechanism mediated by muscarinic receptors, resulting in a stimulation of nicotinic receptors. All the above-mentioned results suggest the major role of parasympathetic-sympathetic balance and of intact sympathetic nerve terminals in the induction of desensitization by muscarinic receptor antagonists.

In summary, in anesthetized dogs, some pharmacological properties of methyl-quinuclidinyl-benzylate differ from those of atropine. The proposed mechanism, which remains hypothetical, is that methyl-quinuclidinyl-benzylate may induce the release of norepinephrine, which provokes a rapid (< 2 h) decrease in the number of externalized myocardial β-adrenoceptors. This unexpected effect may have clinical implications in PET studies, since methyl-quinuclidinyl-benzylate competes with acetylcholine at the post-synaptic level but also probably acts on norepinephrine release and indirectly on myocardial β-adrenoceptors.

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