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CHARACTERIZATION OF MUSCARINIC CHOLINERGIC RECEPTORS IN INTACT MYOCARDIAL CELLS *IN VITRO*

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Abstract—Muscarinic acetylcholine receptors (mAChR) were studied on heart cells grown in culture by the radioligand binding technique. We used [^{3}H]*n*-methyl-scopolamine to monitor the level of receptors on intact cardiocytes. The number of mAChR was very low during the first days in culture (23 fmol/dish). It increased gradually until it reached a plateau on the 4th day (180 fmol/dish), where it remained for 1–2 weeks. To determine whether contractile activity affected the level or affinity of mAChR, the cardiocytes were exposed to agents that stimulate or arrest the heart beat. Treatment with triiodothyronine (T_3, 10–90 nM) for 48 hr caused a reduction in the level of the receptors by 20–30% without changing significantly the affinity of the receptors. Similarly, electrical stimulation caused a reduction in the level of the receptors by 30–40%, without a significant influence on creatine kinase activity. When the myocardial cells were treated with Ca-channel blocker such as metoxyverapamil (D600) (10–30 μg/mL) or diltiazem (10–25 μg/mL) the level of the receptors was also reduced by 30–40%. The reduction in the receptor binding sites was accompanied by an increase in K_d from 0.8 to 3.2 nM in D600-treated cells, whereas there was no significant change in the radioligand affinity after application of diltiazem. Treatment with D600 or T_3 together with cycloheximide showed that under these experimental conditions the rate of receptor degradation was accelerated. The half-life of the receptors in the control was 27 hr, whereas the half-lives of T_3 and D600 were 15 and 18 hr, respectively. It is concluded that regulation of the amount of cholinergic receptors occurs at the level of receptor breakdown, and simple linkage does not exist between the rate of cardiac contractions and the number of mAChR.

Both the contractile force and heart rate are decreased by the binding of the neurotransmitter acetylcholine to muscarinic acetylcholine receptors (mAChR†). The differences in biochemical and pharmacological properties of muscarinic receptors led to the concept of muscarinic receptor subtypes. There are currently three pharmacologically identified subtypes of muscarinic receptors [1]; however, molecular biology approaches have demonstrated the existence of at least five different muscarinic receptor sequences [2]. Heart cells contain only one subtype: M2, m2 [1, 3–6]. The quantification of cardiac mAChR and its interaction with various ligands has been determined using classical receptor binding techniques on tissue homogenates and membrane preparations. While broken cells may possess different receptor properties than intact cells [7–9], the study of mAChR on intact myocytes appears to be more relevant since it is closer to normal physiological conditions.

There are only a few reports on mAChR where the receptors were studied in cardiac cells in culture and most of them employed the lipophilic ligand [^{3}H]quinuclidinyl benzilate. This ligand was not suitable for studying changes in cell surface muscarinic receptors as it readily diffused through the cell membrane. Therefore, studies on mAChR were limited to cell homogenate or membrane preparations [10, 11]. Presently, a hydrophilic muscarinic receptor antagonist [^{3}H]NMS is available which allows labelling of the receptors on intact cardiomyocytes. We describe here the binding properties of mAChR in intact rat cardiomyocytes grown in cell cultures using the water-soluble antagonist [^{3}H]NMS. The radioligand binding assay has been used to study the possible mechanisms involved in the process of receptor down or up regulation as a result of treatment with agents affecting contractile activity. We show that T_3 and ES, which accelerated the rate of contractions, or Ca-channel blockers, which produced contractile arrest, caused a reduction in the number of cholinergic receptors in heart cells. The data indicates that contractile activity does not play a major role in controlling directly the level of mAChR.

MATERIALS AND METHODS

Preparation of heart cell cultures. Rat hearts (1–2 days old) were removed under sterile conditions and washed three times in PBS to remove excess blood cells. The hearts were minced to small fragments and then agitated gently in a solution of proteolytic enzyme-RDB (Ness-Ziona, Israel) prepared from a fig tree extract. The RDB was diluted 1:50 in Ca^{2+}- and Mg^{2+}-free PBS, at 25° for a few cycles of 10 min each, as described previously [12–14]. Medium

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† Abbreviations: CK, creatine kinase; D600, metoxyverapamil; ES, electrical stimulation; [^{3}H]NMS, [^{3}H]*n*-methyl-scopolamine; mAChR, muscarinic acetylcholine receptors, PBS, phosphate-buffered saline; TH, thyroid hormone; T_3, triiodothyronine.

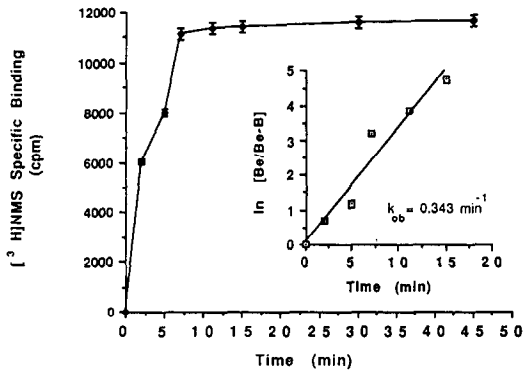

Fig. 1. Time-course of [^{3}H]NMS binding to heart cells. Six-day-old cardiocytes were incubated in the presence or absence of 10^{-4} M NMSBr at 25°. Specific binding was determined as described in Materials and Methods. Each point is the average ± SE of triplicate determinations in sister cultures. Inset: pseudo first order kinetic plot of [^{3}H]-NMS binding. *B*, amount of [^{3}H]NMS bound at each time; *Be*, amount of [^{3}H]NMS bound at equilibrium.

containing 10% horse serum (Biolab, Jerusalem, Israel) was added to supernatant suspensions containing dissociated cells. The mixture was centrifuged at 150 *g* for 5 min. After centrifugation, the supernatant phase was discarded and cells were resuspended in high glucose (5 mg/mL) Dulbecco's modified Eagle's medium (Gibco, Uxbridge, U.K.) supplemented with 10% heat-inactivated horse serum and 2% chick embryo extract. The suspension of cells was diluted to 1.2×10^6 cells/mL and 1.5 mL were placed in 35-mm collagen–gelatin-coated plastic culture dishes. The cultures were incubated in a humidified atmosphere of 10% CO_2, 90% air at 37°. Confluent monolayers, which exhibit spontaneous contractions, developed in culture within 2–3 days. The growth medium was replaced every 3–4 days.

Ligand binding. Intact cells were incubated at room temperature (22–25°) for 30 min, with various concentrations of [^{3}H]NMS, in PBS at pH 7.4. Incubation was stopped by quickly rinsing the cells five times with cold (4–10°) PBS. The cells were dissolved in 0.3 mL Triton X-100 (1%) and radioactivity was determined by scintillation counting. Non-specific binding of [^{3}H]NMS was defined as the amount of radioactivity remaining after incubation with non-radioactive NMSBr (10^{-4} M). Specific [^{3}H]-NMS binding was calculated as the total radioactivity bound minus the non-specific binding (less than 10%).

For calculation of the association rate constant/ k_1, specifically bound [^{3}H]NMS (7 nM) was determined as a function of time as indicated in the graph in (Fig. 1).

In experiments studying the dissociation of specific [^{3}H]NMS binding, cells were incubated at 21° with 7 nM [^{3}H]NMS until equilibrium (30 min). At time zero, the binding mixture was replaced by PBS (1 mL) containing NMSBr (10^{-4} M). The experiment was terminated as in the ligand binding method. k_{off} was calculated from the slope of the plot.

For the competition experiments, cardiomyocytes were incubated in a mixture containing the competing drug at various concentrations and 7 nM [^{3}H]NMS for 30 min at room temperature.

ES. Cardiomyocytes grown for 4 days in culture were transferred to a temperature of 30° (instead of 37°), to reduce the rate of spontaneous contractions for ES. ES of heart cells was achieved by placing a pair of platinum electrodes in the culture medium and fastening them to a cover glass on the 35-mm dish, 25 mm apart, as described previously [15]. The frequency of the pulses was 4 Hz, the duration time 1.0 msec and the voltage, 20 V. After ES, the level of the mAChR was determined by binding experiments.

Protein content and CK activity. Protein determination was performed according to the method of Lowry *et al.* [16], using bovine serum albumin as a standard.

The cardiomyocytes were washed with cold PBS and the cells were homogenized in the same buffer. CK was measured using CK kit (Biotrol, France) and the NADPH produced by the enzyme was measured spectrometrically at a wavelength of 340 nm, as described previously [17].

All drugs were dissolved in PBS except T_3 (1 mM) which was prepared in 0.1 N NaOH. The drugs and chemical used were from the Sigma Chemical Co. (St Louis, MO, U.S.A.) except for [^{3}H]NMS, sp. act. 83 Ci/mmol, which was purchased from Amersham International (Amersham, U.K.).

RESULTS

Characterization of mAChR

Kinetics of [^{3}H]*NMS binding.* [^{3}H]NMS has been shown previously to be an appropriate ligand for binding mAChR on the surface membrane of intact cells [10, 18, 19]. The time-course of specific [^{3}H]-NMS binding to intact cardiomyocytes grown in cell culture is illustrated in Fig. 1. The binding reached equilibrium within 15 min of incubation and was maintained for an additional 90 min (not shown). The rate constant for the pseudo first order association reaction k_{ob} was calculated to be 0.343 min^{-1} (Fig. 1, inset). The binding of [^{3}H]NMS was readily reversible and temperature and dose dependent. At 21° 50% of the ligand was dissociated from the receptors within 8 min (Fig. 2A), whereas at 4° it took at least five times longer (data not shown). The first order kinetic constant for the dissociation reaction k_{off} was calculated to be 0.061 min^{-1} at room temperature (Fig. 2B).

The second order rate constant for the association reaction k_1, was calculated from the equation $k_1 = (k_{ob} - k_{off})/([^3\text{H}]\text{NMS})$ and found to be 4×10^{-7} M^{-1} min^{-1}. Thus, the equilibrium dissociation constant determined kinetically from the ratio k_{off}/k_1 was estimated to be 1.5 nM.

Saturability of [^{3}H]*NMS binding.* To define the saturability of the ligand, intact cardiomyoblasts (6 days old) were incubated at room temperature with various concentrations of [^{3}H]NMS in the presence or absence of cold NMSBr (10^{-4} M). Figure 3 shows the relationship between the radioligand concentrations and the number of specific binding

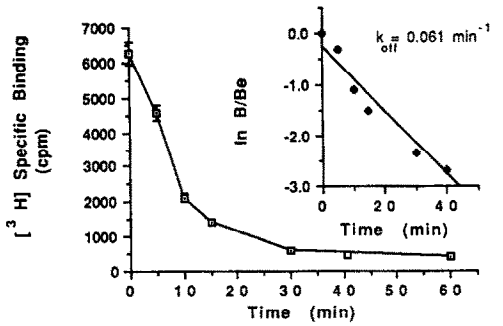

Fig. 2. Dissociation curve of [^{3}H]NMS binding from heart cells. Reverse kinetics of dissociation of [^{3}H]NMS from cardiocytes (6 days old). Specific binding at 21° was determined at subsequent time intervals. After 30 min of incubation the binding mixture was removed and PBS containing NMSBr (10^{-4} M) was added. Each value is the mean ± SE of triplicate determinations from a representative experiment. Inset: first order kinetic plot of dissociation of [^{3}H]NMS bound at each time after dilution of the binding mixture. *Be*, amount of [^{3}H]NMS bound at time 0. The dissociation constant $k_{off} = 0.061$ min^{-1}.

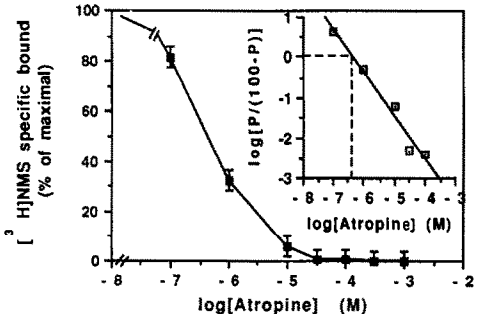

Fig. 4. Inhibition of [^{3}H]NMS binding to heart cells. Five-day-old cardiocytes were incubated in the presence of [^{3}H]-NMS (7 nM) and increasing concentrations of atropine. After 30 min of incubation, at room temperature, specific binding was estimated as described in Materials and Methods. The results are expressed as the percentage of maximum [^{3}H]NMS specifically bound. Data points represent means ± SE of triplicate determinations from a representative experiment. Inset: analysis of the data according to Brown and Hill [22]. *P*, percentage of maximal radioligand bound at equilibrium.

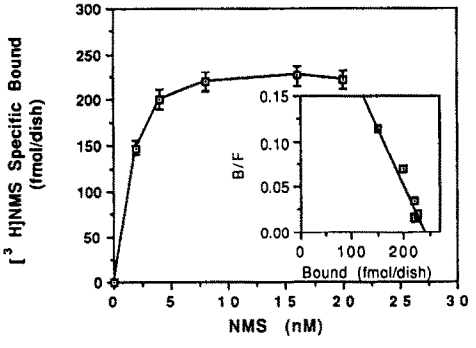

Fig. 3. Specific binding of [^{3}H]NMS to heart cells. Six-day-old rat cardiocytes were exposed to the indicated concentrations of the radioligand as described in Materials and Methods. Specific binding was determined as the [^{3}H]-NMS binding displaceable by 10^{-4} M NMSBr. Data points represent means ± SE of triplicate determinations from a representative experiment of sister cultures. Inset: scatchard plot of the specific [^{3}H]NMS binding. The K_d for [^{3}H]NMS was 0.8 ± 0.09 nM and the maximal binding capacity was 245 ± 43 fmol/dish (the average amount of protein/dish was 0.7 mg).

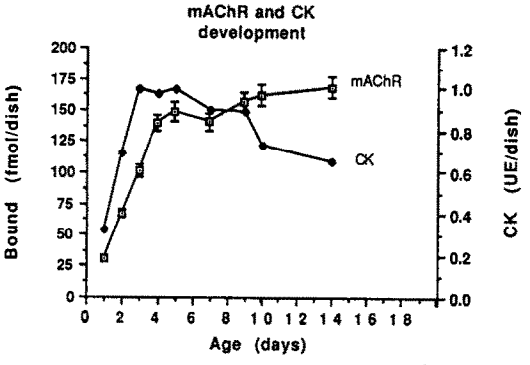

Fig. 5. Age dependence of specific binding of [^{3}H]NMS to heart cells in comparison with CK activity. The level of [^{3}H]NMS binding and CK activity were determined at the indicated time on sister cultures according to Materials and Methods. The results are expressed as means ± SE of triplicate determinations from a representative experiment.

sites in the cultured cardiac cells. The maximal saturation of the antagonist occurred at a concentration of 7 nM. Scatchard analysis [20] of [^{3}H]-NMS binding shows that the maximum number of binding sites was 245 ± 43 fmol/dish (340 fmol/mg protein) and the K_d of [^{3}H]NMS was 0.8 ± 0.09 nM (Fig. 3, inset). The data are best fit by a straight line which indicates that there is one binding site on the receptor.

Competition binding assays were used to examine the binding properties of mAChR. Atropine inhibits [^{3}H]NMS binding with an IC_{50} of 4×10^{-7} M and the calculated K_i (according to Ref. 21) was found to be 4.1×10^{-8} M (Fig. 4). Analysis of the data gave a Hill coefficient [22] of 1.06 (Fig. 4, inset), again indicating one binding site for the receptors.

Development of the receptors. [^{3}H]NMS binding was measured in heart cells during their development in culture. Figure 5 shows that the number of mAChR reached a plateau in 4-day-old cultures (150–200 fmol/dish) which was maintained for up to 2 weeks, whereas the development of CK activity in the heart cells reached a plateau in 3–4-day-old cells (Fig. 5). These results suggest that the cells reach maturity after 4 days *in vitro*, both for CK activity and mAChR level. However, the cells have begun

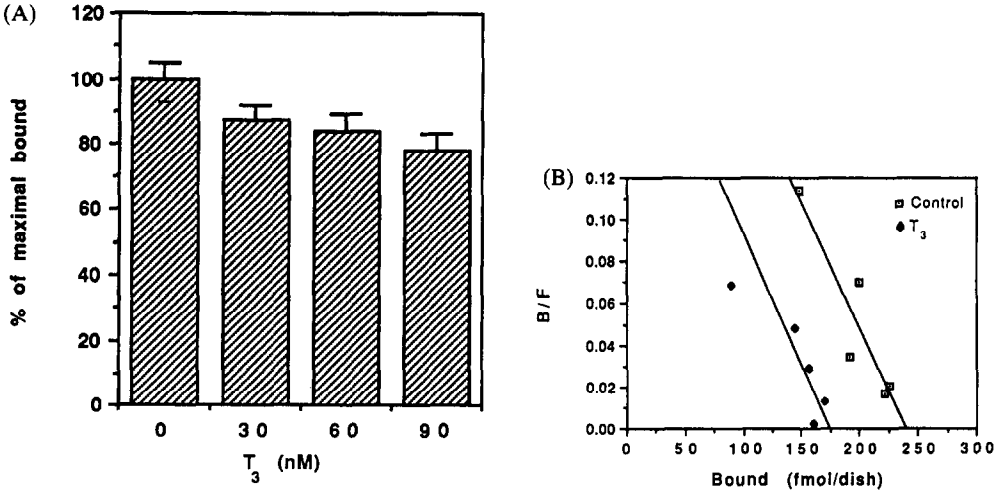

Fig. 6. Effect of T_3 on mAChR level in heart cells. (A) Four-day-old cardiocytes were treated with various concentrations of T_3. The level of mAChR was determined 48 hr later as described in Materials and Methods. (B) Scatchard plot of specific binding of [^{3}H]NMS following 48 hr of treatment with 90 nM T_3 of heart cells. K_d = 0.8 nM, B_{max} = 240 and 175 fmol/dish in control and hormone-treated cells, respectively.

to contract already after 1–2 days in culture. According to these observations, the contraction of the cells and the development of CK and mAChR are independent parameters.

Regulation of mAChR level

Effect of THs on mAChR. Since THs stimulate the contraction of cardiomyoblasts [12, 23], we investigated whether this increase in the rate of heart contractions is associated with a change in the level of mAChR. Thus, myocardial cells were treated with T_3 for 48 hr. The dose–effect of T_3 on mAChR in myocardial cells is shown in Fig. 6A. The maximal decrease in muscarinic receptors was achieved at 10^{-7} M of T_3. Higher concentrations caused toxic effects as revealed by a decrease in the activity of CK. The saturation binding curve of [^{3}H]NMS for the two groups of cells shows a decrease of 25 ± 7% in [^{3}H]NMS binding following T_3 treatment in comparison with the control. Scatchard analysis of 90 nM T_3 indicates that the maximum number of binding sites B_{max} was 175 ± 13 fmol/dish (250 fmol/mg protein) in T_3-treated cells and 240 ± 45 fmol/dish (350 fmol/mg protein) in untreated cells (Fig. 6B). This difference is significant (t = 2.40, 5 df, $P < 0.05$, 1-tailed t-test). The number of binding sites per cell (assuming the receptors are homogeneously dispersed on the cell surface and the number of cells in a dish is 1×10^6) in the control was 145,000 ± 10,500 in comparison to 103,000 ± 7800 in T_3-treated cells. The K_d for [^{3}H]NMS binding was calculated to be 0.8 ± 0.09 nM for the control and T_3-treated cells (insignificant differences were observed between the two groups).

Effect of ES. In order to study whether the increased rate of activity by T_3 caused the reduction in mAChR, the cardiomyocytes were stimulated electrically to contract. To increase the effect of ES,

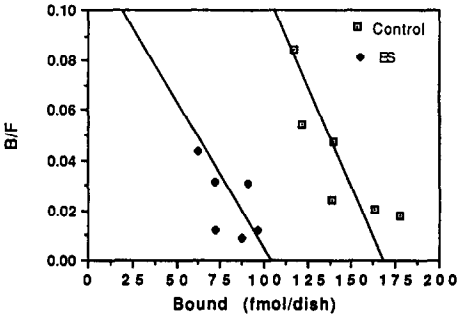

Fig. 7. Effect of ES on the level of mAChR in heart cells. Scatchard plot of specific binding of [^{3}H]NMS following 48 hr of ES (4 Hz). Points are means ± SE of triplicate determinations in a representative experiment. K_d = 0.8 nM, B_{max} = 170 and 103 fmol/dish in control and electrically stimulated cells, respectively.

the temperature of the mature cells (4 days old) was reduced from 37° to 30° during the ES. This reduced the spontaneous rate of contractions from 180 ± 20 to 50 ± 10 beats/min. The level of receptors did not change significantly as a result of a decrease in incubation temperature for up to 48 hr (data not shown). However, ES at 30° reduced the binding sites of [^{3}H]NMS (Fig. 7). Scatchard analysis of the binding shows that ES caused a decrease in receptor number of 40 ± 6% without a significant change in the K_d (0.8 nM). Whereas ES caused a reduction in mAChR level, CK activity did not decrease significantly (Table 1).

Effect of Ca*-channel blockers.* In contrast to ES, which accelerated the rate of contraction and reduced the level of receptors, D600, at doses which inhibited

Table 1. A comparison between CK activity and the level of mAChR

Treatment (48 hr)	CK activity (% of maximal)	B_{max} of mAChR (% of maximal)
Control	100 ± 3	100
T_3 (90 nM)	102 ± 3	75 ± 7
D600 (30 μg/mL)	100 ± 4	66 ± 9
Diltiazem (25 μg/mL)	98 ± 3	62 ± 6
ES	95 ± 2	60 ± 6

The data are (as percentages) the means ± SE of triplicate determinations obtained from different experiments in which the levels of mAChR (B_{max}) and CK activity were compared.

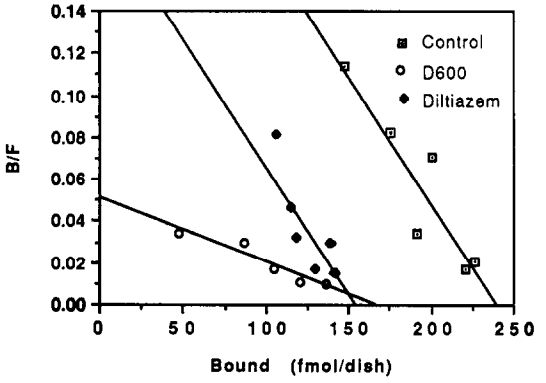

Fig. 8. Effect of D600 and diltiazem on mAChR in heart cells. Four-day-old cardiocytes were treated with either D600 (30 μg/mL) or diltiazem (25 μg/mL) for 48 hr. Scatchard plot of specific binding of [^{3}H]NMS following these treatments yield K_d for the control and diltiazem = 0.8 nM, for D600 = 3.2 nM. B_{max} = 240, 165 and 154 fmol/dish for control, D600 and diltiazem, respectively.

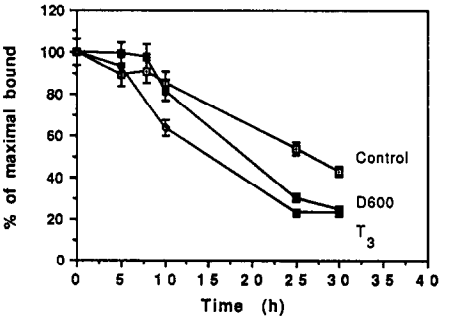

Fig. 9. The rate of degradation of mAChR in heart cells. Four-day-old cardiocytes were treated with cycloheximide (3 μg/mL) and T_3 (90 nM) or D600 (30 μg/mL) and binding of [^{3}H]NMS was performed at the time intervals indicated in the graph. The half-life of the mAChR was found to be 27 hr in the control (□) and 15 hr and 18 hr in T_3 (◆) and D600 (■), respectively.

the rate of spontaneous activity (0.1–1 μg/mL), did not change the receptor level. However, higher doses of D600 (10–30 μg/mL) caused a reduction in [^{3}H]NMS binding (data not shown). Scatchard analysis of 30 μg/mL of D600 for 48 hr shows a decrease of 34 ± 6% in the maximum binding sites for mAChR and a decrease in the affinity of the radioligand to the receptor binding sites from a K_d of 0.8 ± 0.09 to 3.2 ± 0.18 nM (Fig. 8). The reduction in the receptor level was not accompanied by a significant change in CK activity, eliminating toxic effects (Table 1).

When another Ca-channel blocker, diltiazem, was tested on the cardiac cells, it also caused a reduction in [^{3}H]NMS binding. Scatchard analysis of diltiazem treatment (25 μg/mL) for 48 hr shows a reduction of 38 ± 6% without a significant change in the radioligand binding affinity to the receptors (Fig. 8). It is worth mentioning that neither D600 nor diltiazem competed with [^{3}H]NMS binding for mAChR even up to 10^{-4} M of the above Ca-channel blockers (data not shown).

Half-life of mAChR. To study the mechanism by which mAChR are affected, the half-life of mAChR was analysed in heart cells treated with both cycloheximide (a protein synthesis inhibitor) and T_3 or D600. Figure 9 shows that both T_3 and D600 accelerated the rate of degradation of mAChR from 27 hr in the control to 15 and 18 hr, respectively.

DISCUSSION

We have characterized and quantified the surface mAChR in intact cardiomyocytes grown in cultures using the hydrophilic antagonist [^{3}H]NMS. The binding of [^{3}H]NMS is saturable, reversible and of high affinity. The dissociation constant of this ligand is K_d = 0.8 nM (Fig. 3). This value is higher than that obtained by Horackova *et al.* [19], 0.27 nM, measured under similar conditions on intact adult cardiomyocytes, but is closer to the value obtained by Galper *et al.* [10], 0.48 nM, measured on intact chick cultured heart cells. Other groups reported values of K_d from 0.3 to 1.4 nM for [^{3}H]NMS binding to rat hearts [1, 3, 24]. We have calculated the number of receptors per cell, assuming that the receptor distribution is uniform amongst the cells, to be 14.5×10^4 receptors/cardiomyocyte (350–400 fmol/mg protein). A similar density of muscarinic receptors was calculated in intact isolated adult rat

cardiomyocytes [19]. It is also interesting that Nathanson [11] found a similar density of muscarinic receptors in intact chick cultured heart cells, whereas canine sarcolemmal preparation yielded a more than 100 times greater receptor density [25–27].

In our study we have observed a decrease in the [^{3}H]NMS binding (B_{max}) with no significant change in K_d upon exposure of the cardiomyocytes to T_3 (90 nM) for 48 hr. This decrease is in accordance with studies performed *in vivo* [28, 29]. However, in experiments performed *in vivo,* it is difficult to distinguish between a direct effect of the hormone on the heart and indirect effects, mediated by neuronal or other hormones. Thus, experiments performed *in vitro* demonstrate clearly the direct effect of THs on the heart.

We have demonstrated previously that thyroxine induces, in heart cells in culture, a biochemical transition of slow-twitch ("red") muscle to the fast-twitch ("white") muscle type [12]. A similar indication of this transition in the heart is manifested by shortening the duration of action potential following TH treatment [30], and by changing myosin isoenzymes [23, 31]. In fact, the increased heart rate, myocardial contractility and cardiac output in hyperthyroid heart is generally attributed to the increase in the myosin isoenzyme form with higher ATPase activity [32, 33]. However, the myocardial transition from slow to fast type muscle can also explain the increase in myocardial contractility following TH treatment [12]. Furthermore, it has been demonstrated *in vivo* [34] and in culture [13, 35] that THs cause an increase in the number of β-adrenoceptors in heart cells. As muscarinic receptors are antagonistic to adrenoceptors in the heart, we were expecting a decrease in mAChR following TH treatment. This was indeed obtained (Fig. 6).

It is worth mentioning that when T_3 was given to skeletal muscle, it also caused a reduction in the level of nicotinic AChR [36]. This reduction was attributed to the accelerated rate of spontaneous contractions caused by TH, like the effect of ES, which activated the myotubes to contract and also reduced the level of nicotinic AChR [15, 37]. Since THs also stimulate the contraction of cardiomyocytes [12, 23], we compared the hormonal effect with the effect of ES, on the level of mAChR. Indeed ES caused a reduction in [^{3}H]NMS binding similar to that of T_3 (Fig. 7). On the other hand, change in temperature of the growing cells from 37° to 30° which reduced the rate of spontaneous contractions from 180 to 50 beats/min, was not accompanied by a change in the level of mAChR, although the level of the mAChR on control cells at 30° (Fig. 7) was lower than at 37° (175 versus 240 fmol/dish). This difference is probably a result of variation between batches and not of a reduction in temperature since direct comparison of sister cultures did not show this difference (data not shown). Nevertheless, in spite of this decreased level of mAChR in the control group, ES reduced further the level of the receptors (Fig. 7).

Ca-channel blocker D600, at doses of 0.1–1 μg/mL, which inhibits the spontaneous contractions of the cardiomyocytes, did not change the level of the receptors whereas higher doses, 10–30 μg/mL, did reduce [^{3}H]NMS binding. The influence of another Ca-channel blocker, diltiazem, was also analysed. This Ca antagonist belongs to a chemical group, the benzothiazepines, unlike D600 which belongs to the phenylalkilamine group [38]. Both pharmacological agents have specific receptors in the transverse tubular membrane which are voltage-dependent slow Ca-channels (L type). It was found in this study that diltiazem, after 48 hr of treatment, reduced the level of the mAChR as did D600. However, in contrast to D600 there was no significant change in the radioligand affinity for the binding sites. This difference could be a result of variability in the structure and solubility of the two drugs. Thus, we can conclude that there is no simple correlation between the rate of heart beat and the level of mAChR. This conclusion is in contrast with skeletal muscle in which inhibition of the rate of spontaneous contraction either by D600 or TTX increased the level of the receptors, whereas an accelerated rate of myotubes contractions reduced the level of the receptors [15, 36, 37, 39].

There is a lack of correlation between the effects of ES and channel blockade on heart contractions and the level of mAChR. The reduction in the number of mAChR following T_3 or ES treatment had already been proposed as being antagonistic to β-adrenoceptors [13]: the number of β-adrenoceptors was increased following T_3 treatment. Therefore, it was expected that mAChR would be reduced by a similar treatment. However, this rationale cannot explain the reduction of the muscarinic receptor level by Ca-channel blockers. One possible explanation why Ca antagonists caused a reduction in the level of the receptors has been suggested previously by other groups [40, 41]. They demonstrated that contractile activity influences the myocyte size and protein content. Marino *et al.* [40] demonstrated that accelerated contractions of neonatal cardiomyocytes increased the size, whereas Samarel and Engelmann [41] reported that contraction arrest, by L-channel blockade, caused a reduction in myocyte size, growth and protein accumulation. These changes in myocyte turnover could explain the lower level of mAChR following Ca-channel blocker treatment. Thus, the common effect on mAChR by the antagonistic treatments may be caused by entirely different mechanisms. [The unchanged level of CK (Table 1) can be explained by its stability and slower turnover].

To study the mechanism by which the reduction in the level of mAChR was achieved we applied a protein synthesis inhibitor, cycloheximide, and measured the half-life of the receptors. It was found under our experimental conditions that a reduction in the level of the receptors occurred through the accelerated rates of receptor degradation. However, this does not rule out the possibility that there are also some changes in the rate of receptor synthesis following treatment with T_3 or Ca-channel blockers or ES. The unsolved problem is why we did not get up-regulation in the receptor levels. Was it because we did not find the required experimental conditions for the induction process or because the cells used were super-crowded with receptors to acetylcholine as they are not innervated?

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