Development of Autonomic Receptors and their Function in the Chick Heart and Influence of Continuous Infusion of Carbachol

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Abstract: The development of heart rate and of muscarinic and β-adrenergic receptors in chick heart ventricles in the last third of *in ovo* ontogenesis has been studied. The development of these two types of autonomic receptors does not proceed in parallel. While muscarinic receptors are stable between days 13 and 17, β-adrenergic receptors significantly decrease in this period. Muscarinic receptors increase their number from the 17th day, and the highest density can be seen after hatching. β-adrenergic receptors, on the contrary, do not change their density between days 17 and 19, but similarly to muscarinic receptors they increase after hatching (the density after hatching is approximately the same as on the day 13 of embryonic development). We have shown previously that stimulation of one receptor type in the system of autonomic receptors in the heart changes not only the appropriate type of receptor but also the density of the other one. We therefore wondered the information about this in the organism in development. Here we show that when we have infused the eggs in developmental period mentioned above there was no cross-regulation and that muscarinic receptors do not down-regulate. Up-regulation of muscarinic receptors was maintained when the eggs were infused by carbachol from the 13th to 14th day of *in ovo* development. This muscarinic receptor up-regulation was accompanied with paradoxical increase in heart rate. These events are not proteinkinase (PKC) dependent, as bisindolylmaleimide (PKC inhibitor) do not prevent these changes.

Introduction

Muscarinic acetylcholine receptors (MR) and β-adrenergic receptors (BAR) belong to G-protein coupled receptor family, and both are subject to desensitization (for review see [1]). Five subtypes of muscarinic receptors (termed M_1–M_5) have been identified in mammals. Molecular cloning studies have shown that each of the five MR is encoded by distinct genes that lack introns in their coding regions. The M_1, M_3, and M_5 receptors preferentially couple to the pertussis toxin-insensitive G_q family of G proteins to mediate stimulation of phospholipase C; the M_2 and M_4 receptors preferentially couple to the pertussis toxin-sensitive G_i/G_o family of G proteins to mediate inhibition of adenylyl cyclase. All five subtypes can also regulate the activity of specific ion channels. The avian myocardium expresses M_2 and M_4 subtype of muscarinic receptors and β_1 and β_2 subtype of adrenergic receptors [2, 3]. In addition to M_2 and M_4 muscarinic receptors, the chick atria and ventricles also express M_3 subtype [18]. Thus, MR in the chick heart represent a well-studied model for the regulation of cardiac function. On the other hand, chicken cardiac BAR show different properties from either β_1- or β_2-adrenoceptors reported in mammalian hearts, and coupling between binding and adenylyl cyclase might be more efficient after hatching [19].

The development of receptor density in the mammalian heart has been extensively studied, but developmental characteristics of autonomic receptors in

avian heart are unclear yet. It has been studied more time but the results are still controversial.

The decrease in β-adrenergic receptor number from the day 9 through day 19 has been demonstrated [4]. The changes in muscarinic receptor density in two heart compartments (atria and ventricles) have been followed by [5] and the authors concluded that the density in atria and ventricles did not differ till 12^{th} day of the development, than the number of receptors increased in atria and was stable in ventricles. On the contrary, another report [6] did not find any difference in the MR density between days 5 and 8, e.g., in days in which [5] described significant changes in MR density. Between day 10 and hatching, [7] have described significant increase in MR density, but they measured the density in the above mentioned days only. As it can be seen from previous paragraph, the systematic investigation of the development of autonomic receptors in chick embryonic heart has not been performed yet.

Deep investigation of activation or inhibition of one receptor system on both two main types of receptors in the heart has not also been performed yet. In some cases [5, 6], the authors followed the changes in receptor density only after repeated application of agonist into window in the egg shell in 12 hours, and 4 days, respectfully.

The model of avian embryo is unique in that it is closed system (with minimum of external influences), with minimal stress influence of drug application, allowing to study development with main similarities to mammalian organism (the heart receptor expression is similar to mammals except of extra M_4 muscarinic receptor expression).

We used the infusion system developed by Sedláček [8], which enables long term, continuous, and accurate (in concentration of infused solution) spontaneous transfer across the *membrana papyracea* to chorioallantoic circulation. The embryos are not exposed to any mechanical, thermal or the other stress, which cannot be excluded in other systems (repeated application, surgical neural lesions, etc.).

Previously, we showed that carbachol is known to decrease the number of muscarinic receptors in adult animals [9] and also in tissue culture [10].

Some our data also indicated that the increase in muscarinic receptor number can be due to increased expression of odd-numbered muscarinic receptor subtypes. As the chick heart expresses the M_3 muscarinic receptors, coupled to Gq/phospholipase C/proteinkinase C signalization pathway, we employed this unique system allowing us to apply proteinkinase inhibitors (PKC inhibitor in that case) to ascertain what muscarinic receptor subtype is affected in the regulation.

In brief, in the present study, we studied the development of muscarinic and β-adrenergic receptors and of heart rate in the last third of *in ovo* development. We followed changes in receptor density after continuous infusion of unspecific

muscarinic agonist carbachol and proteinkinase (PKC) inhibitor bisindolylmaleimide (BIM) into chorioallantoic circulation of chick embryos.

Material and methods

Animals

We used white Leghorn chicks from the 13th embryonic day to hatching (21st day). The embryos were removed from the eggs, decapitated; hearts were removed, washed in cold saline, the ventricles were carefully prepared, all non-muscular parts were removed, the hearts were weighted, and flash frozen. In appropriate day, when the saturation experiments were performed, the hearts were carefully defrost, homogenised in homogenisation medium (HEPES (20 mmol/l) and $MgCl_2$ (1 mmol/l), pH adjusted to 7.4 in amount of 20 mg of tissue per 1 ml.

Infusion apparatus and dosage of carbachol and bisindolylmaleimide

We infused carbachol (10^{-5} to 10^{-3} in infused solution) into the chorioallantoic circulation according to [8] in following intervals: from 13th to 14th; to 16th; to 18th, and to 20th day. Embryos were killed 24 hours after the end of infusion. Continuous infusion was performed via system depicted at Figure 1. Shortly: We created a small window 5×5 mm in the egg shell and the fiber (N° 9 in Figure 1)

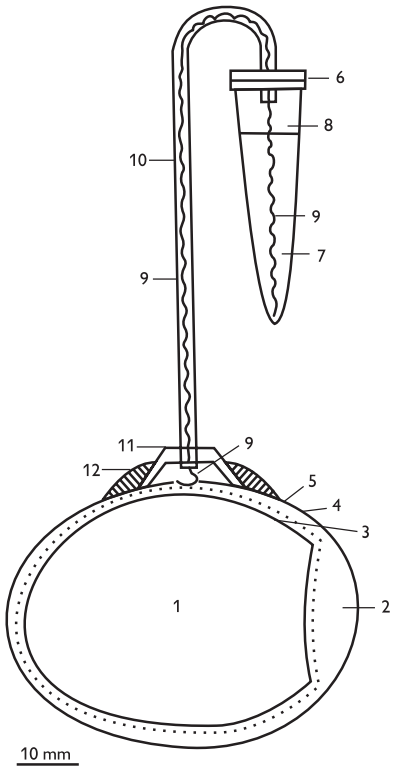

Figure 1 – The infusion apparatus according to [8]. 1 – avian egg, 2 – air space, 3 – chorioallantoic membrane, 4 – paper membrane, 5 – egg shell, 6 – reservoir, 7 – supply of drug, 8 – level of solution (the consumption is regularly measured), 9 – suction fibre, 10 – connecting tube, 11 – fixing foot, 12 – paraffin.

was inserted into the small gut of saline in the window. The apparatus was adjusted to the shell by mixture of stearic acid and parrafin. Twenty-four hours before the end of experiment the infusion apparatus was removed and the hole was covered by medical plaster (Leukopor®). Bisindolylmaleimide was infused using the same apparatus in concentration range from to 10^{-8} to 10^{-5} mol/l.

Saturation binding experiments
Binding experiments were performed similar to previously described [9], [10] using ^{3}H-CGP and ^{3}H-QNB as ligand. The range of concentration was 50–1600, and 12.5–400, respectfully.

Heart rate measurement
Heart rate was measured using acoustocardiography. Briefly, small hole in the eggshell has been made (5×5 mm). The microphone was sealed tightly to the eggshell, the signal was amplified and filtered and the number of peaks per minute was counted using Fourier discrete transformation. In order to eliminate the influence of circadian heart rate fluctuations, the measurements were performed the same daytime for period of 30 minutes in which 10 measurements for 1 minute was received. As a negative control, the eggs without live animal were used.

Material
^{3}H-QNB ((±)-Quinuclidinyl α-hydroxydiphenylacetate, L-[benzilic-4,4'-^{3}H]-) was from Amersham International, Amersham (specific radioactivity 1.54 TBq/mmol), ^{3}H-CGP (-(-)4-(3-tert-butylamino-2-hydroxypropoyx)-[5,73H]benzimidazol-2-one) from NEN, Boston, USA (specific radioactivity 1.24 TBq/mmol), carbamoylcholin chloride from Sigma (St. Louis, USA).

Data treatment
Radioligand binding data were evaluated as described previously [11]. Statistical significance of differences between means was evaluated with one-way, two-way and three-way ANOVA. For multiple comparisons, an adjusted t-test with P values corrected by the Tukey method was used.

Results

Development of receptors
The development of MR and BAR did not proceed in parallel (see Figure 2). Density of MR in heart ventricles from the 13th day to 17th day was stable. The density started to increase from this point and was the highest after hatching. BAR density, on the contrary, decreased to 17th day, was stable between days 17 and 19, but increased between days 19 and 21. The density of BAR after hatching was at the same level as at 13th day of *in ovo* development.

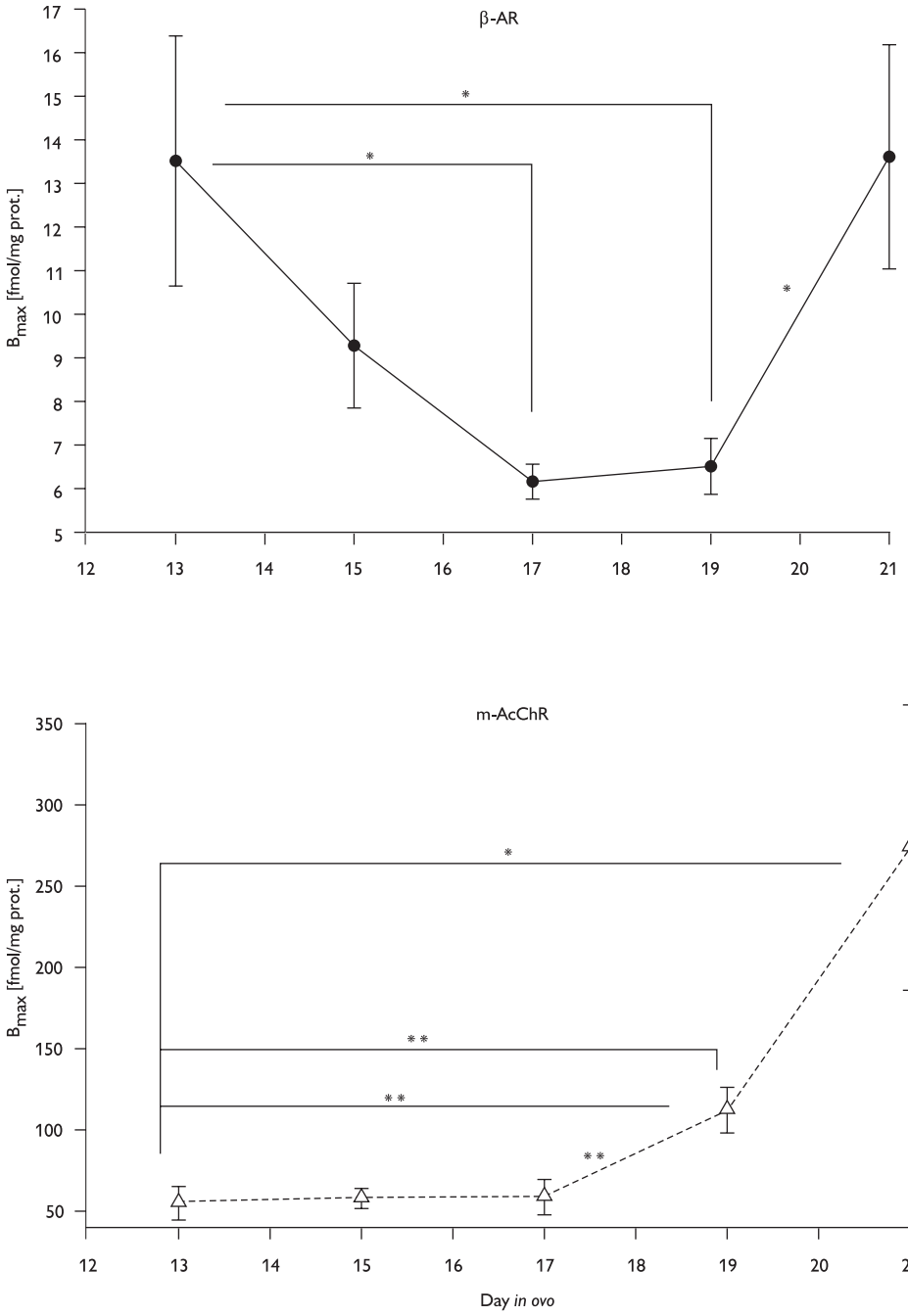

*Figure 2 – Development of BAR and MR in heart ventricles. Abscissa: the age of embryo in days. Ordinate: receptor densities (fmol/mg of protein). *p<0.05, differs from 19th day; # p<0.05, differs from 13th day; ** p<0.05, differs from 15th day; + p<0.05, differs from 17th day.*

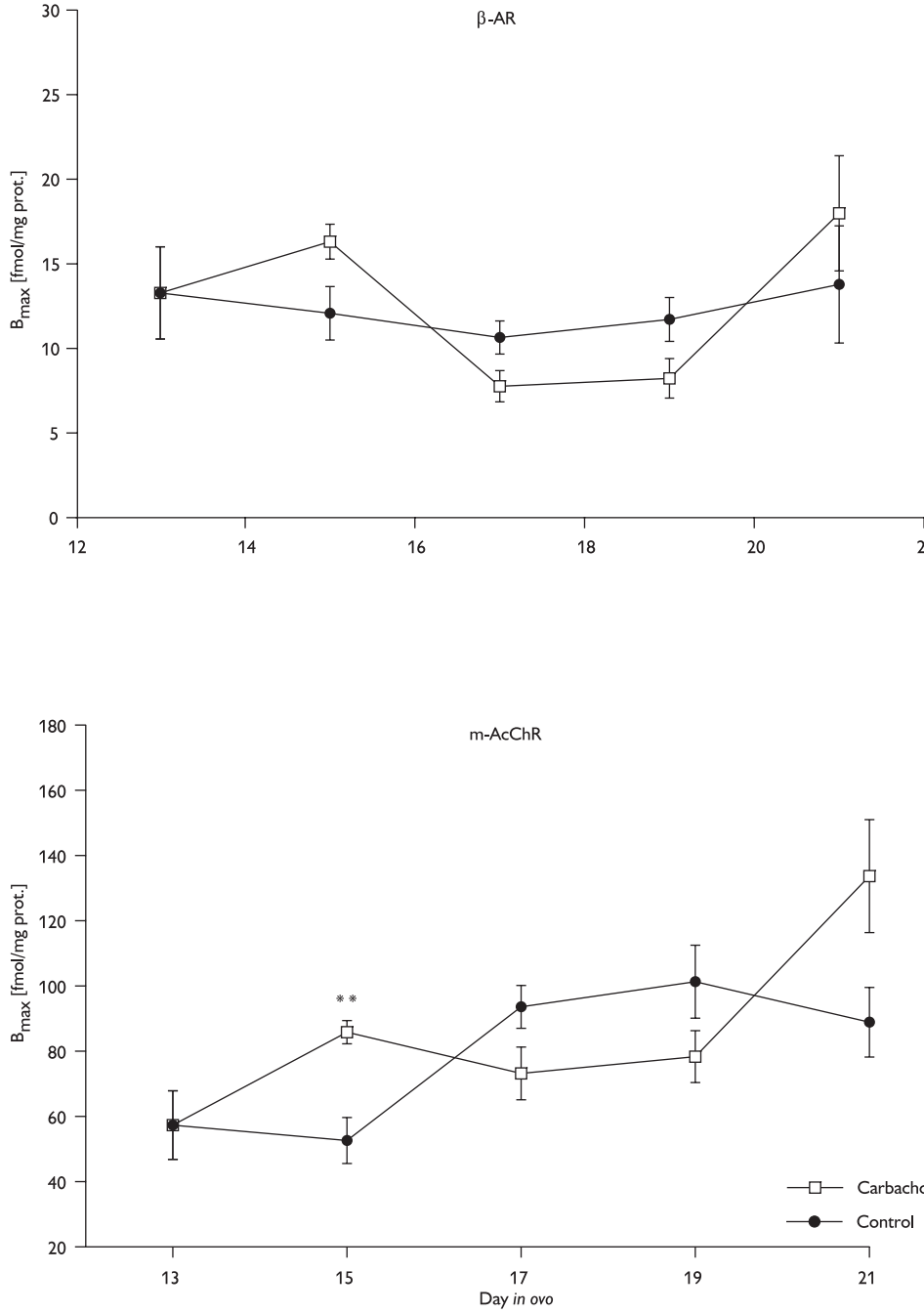

*Figure 3 – Effects of carbachol infusion on receptor densities. b-adrenergic receptors (above), muscarinic receptors (bottom). See legend for explanation. Abscissa: the age of embryo in days. Ordinate: receptor densities (fmol/mg of protein). ** $p<0.01$, different from control.*

Development of heart rate

The heart rate of intact animals is shown as part of Figure 4. The heart rate continuously increased without any interruption from the 13th day to the day before hatching. Then, the decrease of heart rate occurred.

The effects of carbachol and bisindolylmaleimide

Carbachol influenced neither ventricle weight nor lethality of embryos. Average dose of carbachol was 216±14 mg/kg/24 hours. Carbachol changed neither MR nor BAR density in any interval except of the shortest time of infusion, in which it induced a significant increase of MR (Figure 3). This increase was dose dependent (data not shown). Similarly to receptors, the heart rate was influenced during carbachol treatment. We have found paradoxical increase in heart rate in day 14 and 15 of the development (see Figure 4). Before hatching, carbachol caused decrease in heart rate (Figure 4) that was parallel to the increase in muscarinic receptors (Figure 3). In order to elucidate the increase in the heart rate we infused the embryos with proteinkinase C inhibitor bisindolylmaleimide (BIM). Surprisingly, BIM was able to increase the heart rate and that effect was amplified by carbachol (Figure 5).

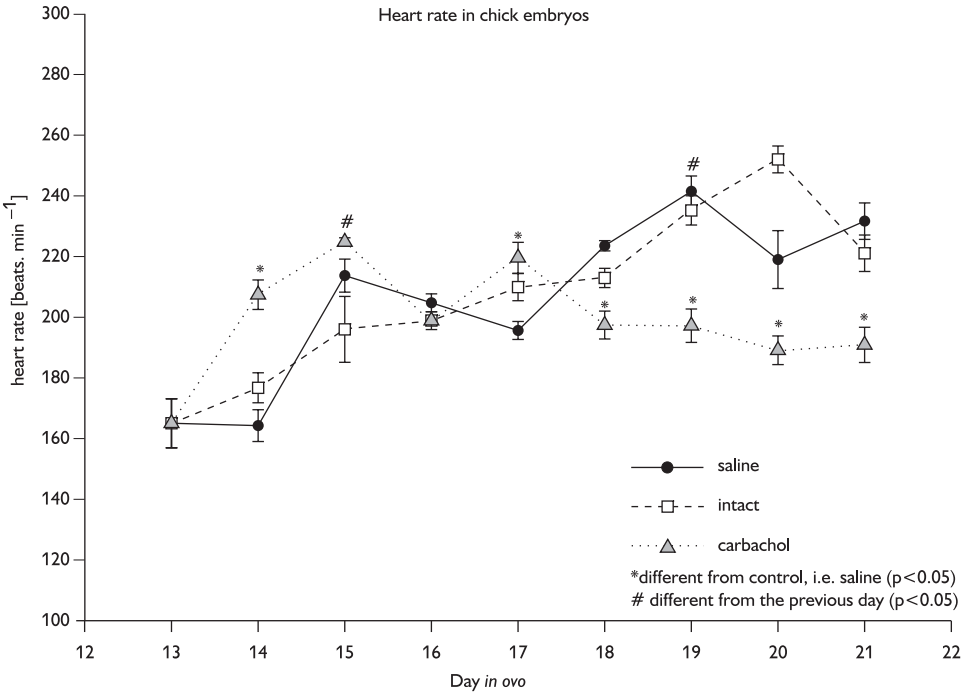

Figure 4 – Heart rate in chick embryos. See legend for explanation. Abscissa: the age of embryo in days. Ordinate: heart rate (beats per minute).

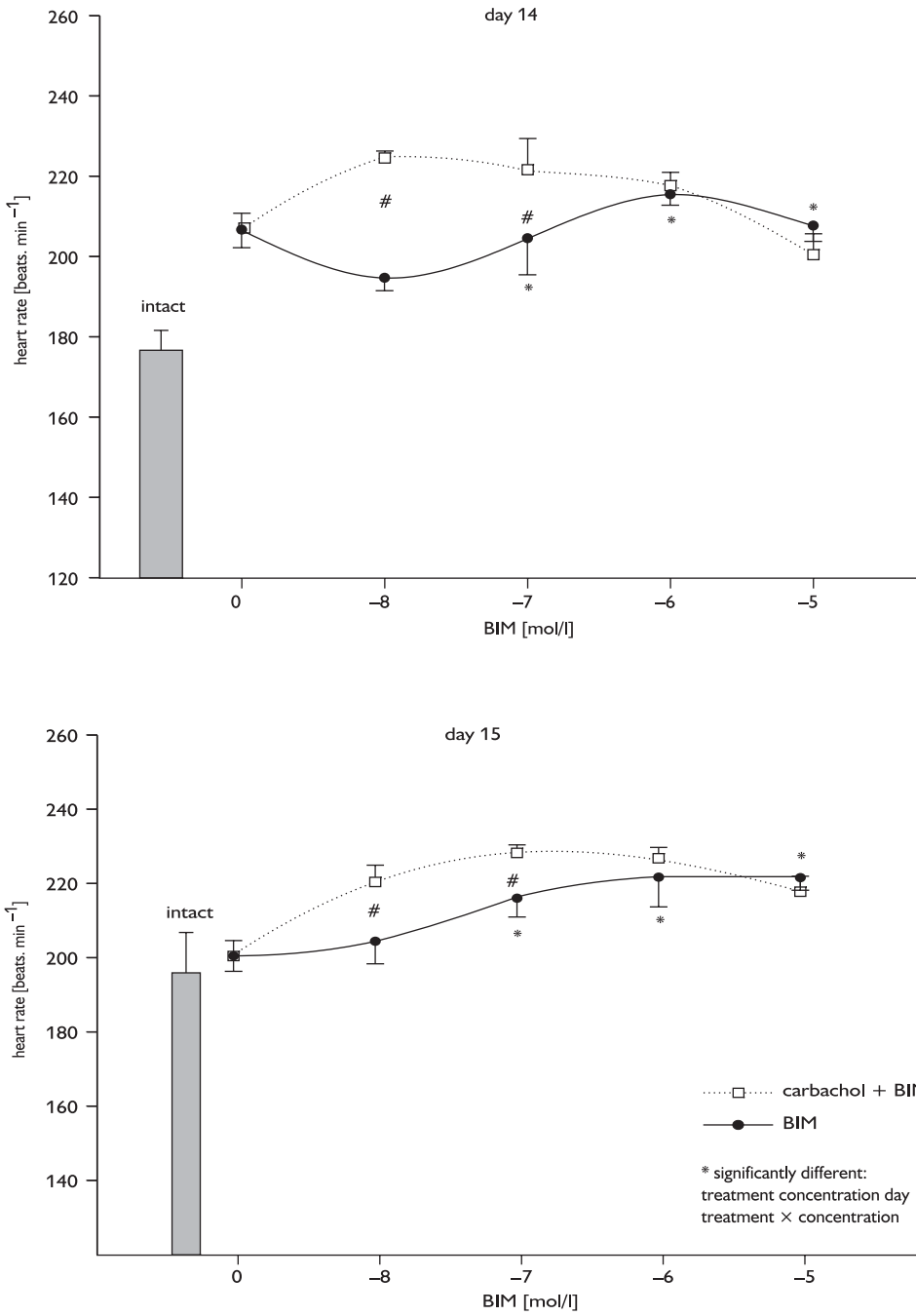

*Figure 5 – The effects of BIM and carbachol on heart rate. Above: 14th day of development. Bottom: 15th day of development. Abscissa: BIM concentration (mol/l). Ordinate: heart rate (beats per minute). *p<0.05 differs from BIM 10-8. #p<0.05 significantly different between carbachol and carbachol+BIM.*

Discussion

Our results showed that in the last third of *in ovo* development:

1) ^{3}H-QNB binding site amount was stable between days 13 and 17, than increased sheer and had peak after hatching
2) ^{3}H-CGP binding site amount decreased significantly between days 13 and 17, than was stable (17th day through 19th day), and increased after hatching.

Continuous carbachol infusion starting at 13th day of *in ovo* development resulted in following events:

1) when infusion lasted 24 hours and animals were killed after 24 hours, ^{3}H-QNB binding sites increased, while ^{3}H-CGP binding sites were not changed significantly,
2) there were no significant differences in receptor number (nor muscarinic nor adrenergic) in longer intervals of infusion,
3) the heart rate paradoxically increased in parallel to changes in muscarinic receptors,
4) the effects on 14th and 15th day was not PKC dependent,
5) moreover, PKC inhibition increased the heart rate.

It is possible to summarize that:

1) both muscarinic receptors and β-adrenergic receptors change their number in the last third of *in ovo* ontogenesis, these changes do not proceed in parallel,
2) the reaction of organism in development to muscarinic stimulation is different from those in adult animals [9], or in cardiomyocytes cultures [10],
3) developing tissue seems to be non-reactive or hyperreactive to these stimuli, respectively,
4) these changes cannot be explained as a consequence of PKC activation.
   Our findings about developmental changes in muscarinic receptors are in good agreement with [5, 7], and developmental changes in β-adrenergic receptors are similar to those described by [4].

Our findings concerning up-regulation of muscarinic receptors after carbachol treatment are new, and differ from previously published data [6, 12, 5], partially from those of [13]. Similar event (non-reactivity to muscarinic stimulation) described [6], in 4-day-old embryos. The decrease in β-adrenergic receptor number from the day 9 through day 19 was demonstrated [4] what is in good agreement to our results. Changes in muscarinic receptor density in two chick heart compartments (atria and ventricles) were followed by [5] and the authors concluded that the density in atria and ventricles did not differ till 12th day of the development, than the number of receptors increased in atria and was stable in ventricles. These findings vary with our results but the explanation of it is difficult. On the contrary, another report [6] did not find any difference in the MR density

between days 5 and 8, e.g., in days in which [5] described significant changes in MR density. Between day 10 and hatching, [7] significant increase in MR density was described, but they measured the density in the above mentioned days only.

The data concerning the regulation of BAR in the avian embryo treated by carbachol are missing. MR were found to be decreased [6] due to carbachol treatment. Moreover, decreased physiological sensitivity mediated by newly synthesized muscarinic acetylcholine receptors in embryonic chicken heart was found [12]. Similar results were obtained in chick cultured cardiomyocytes [15, 16, 17].

The possible explanations for our findings are:

a) "specific plasticity" of developing organism (for review see [14]; homeostatic conditions of embryos can differ from those in cardiomyocytes culture, where the down-regulation has been described [15, 16, 17];
b) different subtype expression in chick myocardium (the expression of M_4 subtype that is not expressed in the mammalian heart);
c) differences in atrial and ventricular muscarinic receptor density.

The other aspect of this phenomenon could be our unique experimental conditions, because we have used continuous infusion, but the others have used dropping of solution into the window only.

In respect to PKC inhibition it is necessary to mention that one can not exclude the possibility that the PKC inhibition is not specifically targeted to the heart tissue and more systems can be affected. Therefore, it is also possible that BIM act on the central parasympathetic nuclei and the increase activity in sympathetic centres is the cause of the heart rate increase. On the other hand, the fact that this increase was potentiated by carbachol makes this possibility improbable.

Our experiments showed that in the last third of chick embryonic development the number of muscarinic and β-adrenergic receptors changed dramatically. The reaction of chick organism to agonist stimulation differs from those described in adult animals or cell cultures. We cannot demonstrate the cross-regulation between muscarinic and β-adrenergic receptors.

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