Developmental changes in β -adrenoceptors, muscarinic cholinergic receptors and Ca^{2+} channels in rat ventricular muscles

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- 1 In an attempt to explain the previous electrophysiological data on the ontogeny of β -adrenergic and muscarinic cholinergic interactions on cardiac Ca^{2+} current, biochemical studies were performed on the ontogeny of β -adrenoceptors, muscarinic cholinergic receptors and Ca^{2+} channels in cardiac muscle of developing rats: 16–20 days old fetuses, 0–20 days old neonates, and 2–3 months old adults.
- 2 Developmental changes in cardiac β -adrenoceptors, muscarinic cholinergic receptors, and Ca^{2+} channels were determined with the use of specific radioligands, [^3H]-dihydroalprenolol (DHA), [^3H]-quinuclidinyl benzilate (QNB), and [^3H]-nitrendipine (NTD), respectively.
- 3 The B_{max} value (fmol mg^{-1} tissue) for [^3H]-DHA binding started to increase on post-gestation day 20, reached almost its maximum level on neonatal day 6, kept almost the same level until neonatal day 20, and then decreased slightly to its adult level.
- 4 The B_{max} value (fmol mg^{-1} tissue) for [^3H]-QNB binding started to increase on post-gestation day 16, reached almost its maximum level on neonatal day 0, remained almost constant until neonatal day 15, and then decreased to its adult level.
- 5 The B_{max} value (fmol mg^{-1} tissue) for [^3H]-NTD binding increased with age between post-gestation day 18 and neonatal day 15, stayed almost constant until neonatal day 20, and then decreased to its adult level.
- 6 The K_d values for [^3H]-DHA, [^3H]-QNB, and [^3H]-NTD bindings remained almost constant during the developmental period examined.
- 7 Isoprenaline (Iso) increased the $\dot{V}_{\text{max}}$ of slow action potentials (APs) from post-gestation day 18, and the adult level was reached at about 2 weeks after birth; this developmental time course is similar to that of Ca^{2+} channels. The number of β -adrenoceptors also started to increase a few days before birth, but attained its peak about one week earlier than did the $\dot{V}_{\text{max}}$ of slow APs or the number of Ca^{2+} channels.
- 8 Acetylcholine (ACh) almost completely abolished the Iso-induced increase in $\dot{V}_{\text{max}}$ observed from post-gestation day 18 to neonatal day 20; this developmental time course for the ACh effect is consistent with the finding that the number of muscarinic cholinergic receptors started to increase on post-gestation day 16 and reached a peak on the day of birth.
- 9 Previous electrophysiological and the present biochemical findings strongly suggest that the functional coupling between muscarinic cholinergic receptors and Ca^{2+} current is already established when the coupling between β -adrenoceptors and Ca^{2+} current starts to operate in developing rat hearts.

Introduction

The development of the neuronal control of the cardiac Ca^{2+} current attracts much attention, because a knowledge of its ontogenesis provides information as to how the complex physiological function of Ca^{2+} currents is gradually put together by the integration of several specific components during development. The main findings from our previous study (Kojima *et al.*, 1990) were: (i) isoprenaline (Iso) increased $\dot{V}_{\text{max}}$ [the maximum rate of rise of action potential (AP)] of slow APs from post-gestation day 18, and its increasing effect reached the adult level about 2 weeks after birth; (ii) acetylcholine (ACh) became effective on post-gestation day 18 in reducing the $\dot{V}_{\text{max}}$ of Iso-induced slow APs to the level before the administration of Iso, and its inhibitory effect became greater as the $\dot{V}_{\text{max}}$ of Iso-induced slow APs increased with age. As described previously (Kojima *et al.*, 1990), the transmembrane signalling mechanism following stimulation of β -adrenoceptors and muscarinic cholinergic receptors by their agon-

ists, which increase and decrease Ca^{2+} current, respectively, depends on the components of the receptors, guanosine triphosphate (GTP)-binding proteins, adenylate cyclase, guanylate cyclase, adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase, and Ca^{2+} channels. Our electrophysiological findings require confirmation by biochemical data on the developmental changes in the components related to β -adrenergic and muscarinic cholinergic interactions on Ca^{2+} current.

Several biochemical studies using rat hearts have addressed the developmental changes in the components mentioned above (see Discussion). Most investigators, however, have examined the developmental changes of only one component. As a result, there are differences in the developmental stages or time periods used and some discrepancies exist among their data even when the same component was examined. The B_{max} values are also often expressed in different units. The 'fmol mg^{-1} protein' unit is most commonly used, but sometimes it is the 'fmol mg^{-1} tissue' unit. The biochemical findings previously reported, therefore, cannot adequately explain our previous electrophysiological data on the developmental

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Figure 1 Analysis of [³H]-dihydroalprenolol (DHA) binding to adult (2–3 months old) rat ventricular muscle. Cardiac membranes were incubated with increasing concentrations of [³H]-DHA ranging from 0.05 to 2.0 nM for 30 min at 25°C: (○), total binding; (□), non-specific binding; (●), specific binding. Non-specific binding was determined in the presence of 1 μM unlabelled propranolol and subtracted from total binding. Duplicate estimations were used for each point. Scatchard analysis of the specific binding is represented in the inset: $B_{\text{max}} = 4.754 \text{ fmol mg}^{-1} \text{ tissue}$ and $K_d = 0.481 \text{ nM}$. The results shown are representative of 6 experiments.

changes in β -adrenergic and muscarinic cholinergic interactions on Ca^{2+} current.

To clarify such discrepancies, we have investigated the developmental changes in β -adrenoceptors and muscarinic cholinergic receptors and in Ca^{2+} channels in rat ventricular muscles by using the specific radioligands [³H]-dihydroalprenolol (DHA), [³H]-quinuclidinyl benzilate (QNB), and [³H]-nitrendipine (NTD), respectively. We have obtained some biochemical data to explain our previous electrophysiological ones, i.e., the developmental changes in Iso-ACh interaction on cardiac slow APs (Kojima *et al.*, 1990).

Methods

Animals and tissue preparations

Foetal (16–20 days post-gestation), neonatal (0–20 days old), and adult (2–3 months old) Wistar rats were used in the present experiments. The method of obtaining foetuses and neonates of specified ages has been described (Kojima *et al.*, 1990). The hearts were rapidly excised and transferred into an ice-cold oxygenated modified Tyrode solution (for composition, see Kojima *et al.*, 1990). Ventricles dissected from the hearts were quickly frozen and kept at -80°C until required for binding assay.

The frozen ventricles were weighed, minced with scissors, and then homogenized in 40 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) with a polytron apparatus ($2 \times 20 \text{ s}$, setting 5: NS-310E, Niti-on, Tokyo, Japan). The homogenates were centrifuged at 40,000g for 20 min at 4°C (18PR-5, Hitachi, Tokyo, Japan). The resultant pellets were washed twice in the ice-cold Tris-HCl buffer. They were finally resuspended in 40 volumes of the buffer and used as tissue homogenates for the following binding assays.

Radioligand binding assays

Binding of [³H]-DHA, [³H]-QNB, and [³H]-NTD to cardiac β -adrenoceptors, muscarinic cholinergic receptors and Ca^{2+} channels, respectively, were assayed by the methods of Yamada *et al.* (1984), Yamamura & Snyder (1974), and Erman *et al.* (1983). Briefly, the tissue homogenates (2.5 mg tissue for [³H]-DHA and [³H]-NTD binding assays and 5 mg tissue for [³H]-QNB binding assay) were incubated with different concentrations of: (i) [³H]-DHA (0.05–2 nM) in a final volume of 1 ml of 50 mM Tris-HCl buffer (pH 7.4) for 30 min at 25°C ; (ii) [³H]-QNB (0.01–0.4 nM) in a final volume of 1 ml of 50 mM Na/K-phosphate buffer (pH 7.4) for 60 min at 25°C ; and (iii) [³H]-NTD (0.05–1 nM) in a final volume of 1 ml of 50 mM Tris-HCl buffer (pH 7.4) for 90 min at 25°C . After a steady state had been reached, the binding reaction was terminated by rapid filtration under reduced pressure through Whatman GF/B glass-fibre filters using a Brandel cell harvester (M-24R, Brandel, MD, U.S.A.). The filters were rinsed immediately three times with 3 ml of the ice-cold assay buffers for [³H]-DHA, [³H]-QNB and [³H]-NTD binding assays.

Tissue-bound radioactivity was extracted from the filters for 12–24 h in 10 ml of scintillation fluid (Emulsifier-Scintillator 299TM, Packard, IL, U.S.A.). The radioactivity was determined in a liquid scintillation counter (TRI-CARB 4640, Packard, IL, U.S.A.) at a counting efficiency of 30–40%. All binding measurements were made in duplicate in 3–8 independent experiments (except 2 experiments for [³H]-DHA and [³H]-QNB bindings on post-gestation day 16). [³H]-DHA, [³H]-QNB, and [³H]-NTD bindings displaced by 1 μM propranolol, 1 μM atropine, and 1 μM nifedipine, respectively, were considered to be specific. All data in the text refer to specific binding. For the [³H]-NTD binding assay, all experiments were carried out under dim light because of the light sensitivity of the dihydropyridine derivative. Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine albumin as the standard.

Data analysis

Binding data were analysed as described previously (Yamada *et al.*, 1984). Receptor or channel binding data from the saturation studies were analysed by a modified Scatchard equation corresponding to the linear equation:

$$B = K_d(B/F) + B_{\text{max}}$$

where B is the specifically bound ³H-ligand, F is the free concentration of ³H-ligand, K_d is the apparent dissociation equilibrium constant, and B_{max} is the total receptor or channel density. When the binding data are plotted in this manner, the negative of the slope of the regression line gives K_d , and the Y-intercept is B_{max} . The curve fitting programme was obtained from General Statistics (Hewlett-Packard, CO, U.S.A.) for use with a microcomputer (HP9825A, Hewlett-Packard, CO, U.S.A.).

Data are expressed as means $\pm$ s.e.mean. A one-way analysis of variance (ANOVA) and Dunnett's test were used for comparison among the means of the different age groups (Wallenstein *et al.*, 1980). A *P* value of < 0.05 was considered statistically significant. Calculations and analysis were done with the aid of a personal computer (PC-9801RX, NEC, Tokyo, Japan).

Drugs and chemicals

[³H]-DHA (specific activity, 95 Ci mmol⁻¹), [³H]-QNB (43.6 Ci mmol⁻¹), and [³H]-NTD (73.7 Ci mmol⁻¹) were purchased from New England Nuclear (Boston, MA, U.S.A.). Propranolol hydrochloride and atropine sulphate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and nifedipine from Yodogawa Seiyaku Co. (Osaka, Japan). Other drugs and chemicals were obtained from commercial sources (Sigma Chemical Co. or Nakarai Chemicals, Kyoto, Japan).

Figure 2 Analysis of [^3H]-quinuclidinyl benzilate (QNB) binding to adult (2–3 months old) rat ventricular muscle. Cardiac membranes were incubated with increasing concentrations of [^3H]-QNB ranging from 0.01 to 0.4 nM for 60 min at 25°C: (○), total binding; (□), non-specific bindings; (●), specific binding. Non-specific binding was determined in the presence of 1 μM unlabelled atropine and subtracted from total binding. Duplicate estimations were used for each point. Scatchard analysis of the specific binding is represented in the inset: $B_{\text{max}} = 21.80 \text{ fmol mg}^{-1} \text{ tissue}$ and $K_d = 0.0657 \text{ nM}$. The results shown are representative of 6 experiments.

Figure 3 Analysis of [^3H]-nitrendipine (NTD) binding to adult (2–3 months old) rat ventricular muscle. Cardiac membranes were incubated with increasing concentrations of [^3H]-NTD ranging from 0.05 to 1.0 nM for 90 min at 25°C: (○), total binding; (□), non-specific binding; (●), specific binding. Non-specific binding was determined in the presence of 1 μM unlabelled nifedipine and subtracted from total binding. Duplicate estimates were used for each point. Scatchard analysis of the specific binding is represented in the inset: $B_{\text{max}} = 11.31 \text{ fmol mg}^{-1} \text{ tissue}$ and $K_d = 0.216 \text{ nM}$. The results shown are representative of 7 experiments.

Figure 4 Developmental changes in B_{max} for specific binding of [^3H]-dihydroalprenolol (DHA) (β -adrenoceptors) to rat cardiac cell membrane. The B_{max} values are expressed in units of $\text{fmol mg}^{-1} \text{ tissue}$ (a) and of $\text{fmol mg}^{-1} \text{ protein}$ (b). Column heights represent mean values and vertical bars show s.e.mean of 2–6 separate determinations from rats at various ages. The number of experiments is given at the bottom of columns. On the abscissae, F and N denote foetal and neonatal age, respectively. These abbreviations are used in subsequent legends. * Significantly different from the highest value on neonatal day 15 (a) or that on neonatal day 6 (b), $P < 0.05$, Dunnett's test.

Results

Equilibrium bindings of [^3H]-DHA, [^3H]-QNB, and [^3H]-NTD to heart homogenates

Radioligands of [^3H]-DHA, [^3H]-QNB, and [^3H]-NTD were used as biochemical markers for the differentiation of β -adrenoceptors, muscarinic cholinceptors and Ca^{2+} channels, respectively. In preliminary experiments, we confirmed that ligand binding to heart homogenates reached equilibrium at 25°C by 30 min for [^3H]-DHA, 60 min for [^3H]-QNB, and 90 min for [^3H]-NTD under the present experimental conditions. The main panels of Figures 1, 2, and 3 depict the total, specific, and non-specific bindings of [^3H]-DHA, [^3H]-QNB, and [^3H]-NTD, respectively, to the homogenates of adult rat ventricles. The specific binding, i.e., the difference between the total and non-specific bindings, is a saturable process. Specific bindings of [^3H]-DHA, [^3H]-QNB, and [^3H]-NTD at a saturable concentration were $28 \pm 6\%$ (at 0.5 nM, $n = 6$), $95 \pm 1\%$ (at 0.1 nM, $n = 6$), and $47 \pm 2\%$ (at 0.2 nM, $n = 7$), respectively.

The insets in Figures 1, 2, and 3 show Scatchard plots for the specific bindings of [^3H]-DHA, [^3H]-QNB, and [^3H]-NTD, respectively, to their receptors or channels. The linear curve of Scatchard plots demonstrates the presence of a single class of binding site for each ligand under the present conditions. This is also true of the homogenates from foetal and neonatal ventricles (data not shown).

Developmental changes in [^3H]-DHA binding sites (β -adrenoceptors)

The density and affinity of β -adrenoceptors for [^3H]-DHA were determined by computing B_{max} (the maximum number of

Table 1 Developmental changes in K_d values for [^3H]-dihydroalprenolol (DHA), [^3H]-quinuclidinyl benzilate (QNB), and [^3H]-nitrendipine (NTD) binding to rat cardiac cell membrane at various ages

Age (days)	[^3H]-DHA binding	K_d values (nM) [^3H]-QNB binding	[^3H]-NTD binding
16 F	0.227 $\pm$ 0.094 (2)	0.075 $\pm$ 0.005 (2)	0.175 $\pm$ 0.029 (4)
18 F	0.391 $\pm$ 0.099 (5)	0.111 $\pm$ 0.013 (4)	0.145 $\pm$ 0.033 (5)
20 F	0.378 $\pm$ 0.081 (5)	0.107 $\pm$ 0.003 (5)	0.147 $\pm$ 0.011 (7)
0 N	0.354 $\pm$ 0.059 (6)	0.138 $\pm$ 0.011 (6)	0.153 $\pm$ 0.021 (7)
3 N	0.352 $\pm$ 0.056 (3)	0.128 $\pm$ 0.012 (4)	0.186 $\pm$ 0.014 (6)
6 N	0.444 $\pm$ 0.047 (4)	0.113 $\pm$ 0.009 (5)	0.214 $\pm$ 0.019 (6)
10 N	0.378 $\pm$ 0.047 (5)	0.118 $\pm$ 0.011 (5)	0.198 $\pm$ 0.009 (7)
15 N	0.385 $\pm$ 0.040 (5)	0.122 $\pm$ 0.012 (5)	0.233 $\pm$ 0.015 (7)
20 N	0.430 $\pm$ 0.064 (6)	0.102 $\pm$ 0.005 (6)	0.239 $\pm$ 0.014 (6)
Adults	0.413 $\pm$ 0.064 (6)	0.094 $\pm$ 0.011 (6)	0.212 $\pm$ 0.012 (7)

K_d values were obtained from the Scatchard analysis of saturation experiments. Values are means $\pm$ s.e.mean of 2–7 preparations in each group (figures in parentheses give the number of experiments). ANOVA indicates that there are no significant differences in K_d values of each ligand group ($P > 0.05$). 'F' and 'N' in the age column mean foetal and neonatal age, respectively.

binding sites) and apparent K_d (dissociation constant) values, respectively, from Scatchard plots of binding data obtained. Developmental changes in B_{max} and K_d for [^3H]-DHA binding were studied in foetal (16–20 days old), neonatal (0–20 days old), and adult (2–3 months old) rat ventricles.

Figure 4 gives a summary of the present results obtained for [^3H]-DHA binding ($n = 2-6$). The time courses of developmental changes in B_{max} are different when the B_{max} values are expressed in units of fmol mg^{-1} tissue and of fmol mg^{-1} protein. In the case of the ' fmol mg^{-1} tissue' unit (Figure 4a), the B_{max} value started to increase steeply on post-gestation day 20, reached almost a maximum level on neonatal day 6, kept almost the same level until neonatal day 20, and then decreased slightly to its adult level. In the case of the ' fmol mg^{-1} protein' unit (Figure 4b), the B_{max} value increased steeply with age between post-gestation day 18 and neonatal day 6, and then decreased moderately to its adult level.

In Table 1, developmental changes in K_d values for [^3H]-DHA binding are summarized. Analysis of variance of the data given in Table 1 shows that there are no significant differences in the apparent K_d values for specific [^3H]-DHA binding among the different age groups examined ($P > 0.05$, ANOVA, $n = 2-6$).

Developmental changes in [^3H]-QNB binding sites (muscarinic cholinceptors)

[^3H]-QNB binding was examined as a measure of muscarinic cholinceptors. Developmental changes in B_{max} and K_d for [^3H]-QNB binding were also examined in rat hearts of the same pre- and post-natal ages as those in the case of [^3H]-DHA binding assay.

Figure 5 gives a summary of the developmental changes in B_{max} for [^3H]-QNB binding ($n = 2-6$). Their developmental time courses are also different when the B_{max} values are expressed in units of fmol mg^{-1} tissue and of fmol mg^{-1} protein. In the case of the ' fmol mg^{-1} tissue' unit (Figure 5a), the B_{max} value showed a steady-state increase from post-gestation day 16, reached almost a maximum level on neonatal day 0, remained almost constant until neonatal day 15, and then decreased slightly to its adult level. In the case of the ' fmol mg^{-1} protein' unit (Figure 5b), the B_{max} value increased with age between days 16 and 20 post-gestation, kept almost a constant level until neonatal day 3, and then decreased greatly to its adult level.

In Table 1, developmental changes in K_d values for [^3H]-QNB binding are summarized. The values of K_d remained

Figure 5 Developmental changes in B_{max} for specific binding of [^3H]-quinuclidinyl benzilate (QNB) (muscarinic cholinceptors) to rat cardiac cell membrane. The B_{max} values are expressed in units of fmol mg^{-1} tissue (a) and of fmol mg^{-1} protein (b). Column heights represent mean values and vertical bars show s.e.mean of 2–6 separate measurements from rats at various ages. The number of experiments is given at the bottom of columns. *Significantly different from the highest value on neonatal day 15 (a) or that on neonatal day 3 (b), $P < 0.05$, Dunnett's test.

almost constant at all developmental stages studied ($P > 0.05$, ANOVA, $n = 2-6$).

Developmental changes in [^3H]-NTD binding sites (Ca^{2+} channels)

[^3H]-NTD binding was studied as a measure of NTD-sensitive Ca^{2+} channels. Developmental changes in B_{max} and K_d for [^3H]-NTD binding were also examined in rat hearts of the same pre- and post-natal ages as those in the case of [^3H]-DHA binding assay.

Figure 6 summarizes the developmental changes in B_{max} for [^3H]-NTD binding ($n = 4-7$). Their developmental time courses are also different when the B_{max} values are expressed in units of fmol mg^{-1} tissue and of fmol mg^{-1} protein. In the case of the ' fmol mg^{-1} tissue' expression (Figure 6a), the B_{max} value increased with age between post-gestation day 18 and neonatal day 15, kept almost the same level until neonatal day 20, and then decreased slightly to its adult level. In the case of the ' fmol mg^{-1} protein' expression (Figure 6b), the B_{max} value started increasing on post-gestation day 16, reached almost a maximum level on neonatal day 6, remained almost constant until neonatal day 20, and then decreased slightly to its adult level.

In Table 1, developmental changes in K_d values for [^3H]-NTD binding are summarized. The values of K_d remained almost constant at all developmental stages studied ($P > 0.05$, ANOVA, $n = 4-7$).

Discussion

Developmental changes in β -adrenoceptors, muscarinic cholinceptors, and Ca^{2+} channels

In the present study (Figure 4), the number of [^3H]-DHA binding sites (β -adrenoceptors; fmol mg^{-1} tissue) started to

Figure 6 Developmental changes in B_{max} for specific binding of [3 H]-nitrendipine (NTD) (Ca^{2+} channels) to rat cardiac cell membrane. The B_{max} values are expressed in units of $fmol\ mg^{-1}$ tissue (a) and of $fmol\ mg^{-1}$ protein (b). Column heights represent mean values and vertical bars show s.e.mean of 4–7 separate determinations from rats at various ages. The number of experiments is given at the bottom of columns. * Significantly different from the highest value on neonatal day 20 (a and b), $P < 0.05$, Dunnett's test.

increase on day 20 post-gestation, reached nearly a maximum level on neonatal day 6, and remained almost constant thereafter. The number of [3 H]-DHA binding sites ($fmol\ mg^{-1}$ protein), however, increased rapidly between post-gestation day 18 and neonatal day 6, and decreased gradually from neonatal day 10 to the adult level. Systematic studies on post-natal development of β -adrenoceptors have been reported, but none on their pre-natal development. Lau & Slotkin (1982) found that specific binding of [3 H]-DHA ($pmol\ g^{-1}$ tissue) approximately doubled from 3 to 28 days after birth, increasing steeply between 3 and 6 days of age and then gradually between 6 and 28 days of age. Noguchi *et al.* (1981) and Noguchi & Whitsett (1983) showed that [3 H]-DHA binding sites ($fmol\ mg^{-1}$ protein) were most numerous at 1 day of age and gradually decreased with age to the adult level. The developmental pattern of [125 I]-pindolol binding sites ($fmol\ mg^{-1}$ protein), however, showed a large increase between neonatal days 5 and 15 followed by a decline between 15 and 40 days of age (Slotkin *et al.*, 1988). Cros *et al.* (1988) reported that the B_{max} of [125 I]-iodocyanopindolol ($fmol\ mg^{-1}$ protein) was half that of adults during neonatal days 0–10 and increased progressively during days 15 to 60. It is clear that there are vast discrepancies among the data in the literature. It is also noted that these ontogenic data on radioligand binding for β -adrenoceptors are incomplete because these data are lacking for the pre-natal developmental changes of age.

In the present study (Figure 5), the number of receptor binding sites for [3 H]-QNB (muscarinic cholinergic; $fmol\ mg^{-1}$ protein) started increasing on day 16 post-gestation, reached nearly a peak level 4 days later, remained almost constant until neonatal day 3, and then gradually decreased to the adult level. Nedoma *et al.* (1986) reported that the density of [3 H]-QNB binding sites ($pmol\ g^{-1}$ protein) increased steeply between the 15th and the 19th days of pre-natal development, and then gradually decreased to the adult level. Ho *et al.* (1986) observed that the number of [3 H]-QNB

binding sites ($fmol\ mg^{-1}$ protein) showed an increase between post-gestation day 16 and the day of birth, remained at this level during the first week of post-natal life, and reached a peak on day 9. This was followed by a gradual decline over the next 6 months. These two groups, however, gave no information about developmental changes in β -adrenoceptors and Ca^{2+} channels, although Ho *et al.* (1986) demonstrated developmental changes in the content of calmodulin.

In the present study (Figure 6), the number of [3 H]-NTD binding sites (Ca^{2+} channels; $fmol\ mg^{-1}$ protein) gradually increased starting on day 16 post-gestation, reached almost a maximum level on neonatal day 6, remained almost constant until neonatal day 20, and then decreased slightly to the adult level. In regard to the developmental changes in Ca^{2+} channels, Kazazoglou *et al.* (1983) reported that the number of [3 H]-NTD receptors ($fmol\ mg^{-1}$ protein) was low in the foetal stage and increased markedly between 17 days pre-natal and 15 days post-natal. Then B_{max} values remained constant between 15 days post-natal and the adult stage (2 months). Our data are essentially similar to those of Kazazoglou *et al.* (1983).

B_{max} values expressed in units of $fmol\ mg^{-1}$ tissue and $fmol\ mg^{-1}$ protein

As is evident from Figures 4–6, developmental time courses of B_{max} for [3 H]-DHA, [3 H]-QNB, and [3 H]-NTD bindings are different when the B_{max} values are expressed in $fmol\ mg^{-1}$ tissue and $fmol\ mg^{-1}$ protein units. In general, the B_{max} values expressed in ' $fmol\ mg^{-1}$ protein' units reached a maximum level earlier in their developmental time courses than did those expressed in ' $fmol\ mg^{-1}$ tissue' units. Furthermore, the former B_{max} values ($fmol\ mg^{-1}$ protein) showed a greater decrease in magnitude from the maximum level to the adult level than the latter B_{max} values ($fmol\ mg^{-1}$ tissue).

These discrepancies between the B_{max} data expressed in different units may be due largely to the following: (i) the ratio of ' $mg\ protein/mg\ tissue$ ' increases with age, i.e., 0.055 ± 0.005 ($n = 6$) on day 16 post-gestation, 0.076 ± 0.003 ($n = 13$) on the day of birth, 0.111 ± 0.004 ($n = 12$) on neonatal day 10, 0.123 ± 0.006 ($n = 11$) on neonatal day 20, and 0.144 ± 0.006 ($n = 12$) at adult ages of 2–3 months (the present data); (ii) the ratio of ' $sarcolemmal\ protein/cell\ protein$ ' or ' $surface\ area/fibre\ mass$ ' decreases with age because of the development of intracellular organs (Baker & Potter, 1980; Whitsett & Darovec-Beckerman, 1981; Nedoma *et al.*, 1986).

The developmental time course of B_{max} for [3 H]-DHA and [3 H]-NTD bindings (present data) and of GTP-binding protein levels (Kojima *et al.*, 1988) are more similar to those of Iso-induced increases in $\dot{V}_{max}$ (Kojima *et al.*, 1990) when the former biological data are expressed in units of $fmol\ mg^{-1}$ tissue than when $fmol\ mg^{-1}$ protein is used. In the following section, therefore, we used the B_{max} data expressed in ' $fmol\ mg^{-1}$ tissue' units for comparison with our electrophysiological data.

A comparison between the present biochemical findings and the preceding electrophysiological findings on the effect of β -adrenergic and muscarinic cholinergic interactions on Ca^{2+} current

Iso increases the $\dot{V}_{max}$ of slow APs gradually from post-gestation day 18 and the adult level is reached about 2 weeks after birth (Kojima *et al.*, 1990). This developmental time course correlates well with that of Ca^{2+} channels (present data) and of stimulatory GTP-binding (G_s) proteins (Kojima *et al.*, 1988). The number of β -adrenoceptors started to increase on post-gestational day 20 when the G_s protein, the number of Ca^{2+} channels, and the $\dot{V}_{max}$ of Iso-induced slow APs also started to increase. The number of β -adrenoceptors, however, reached its peak about one week earlier than did the other three parameters (Figure 7).

Acetylcholine (ACh) almost completely abolished the Iso-induced increase in $\dot{V}_{\max}$ observed on post-gestation days 18–20 and neonatal days 0–20 as well as in adult rats (Kojima *et al.*, 1990). This finding can be explained by the following facts: (i) the density of muscarinic cholinergic receptors is higher than that of β -adrenoceptors and of Ca^{2+} channels during the developmental period examined, and (ii) the number of muscarinic cholinergic receptors reaches a peak 1–2 weeks earlier than does that of β -adrenoceptors, of Ca^{2+} channels (present data) and of G_i protein levels (Kojima *et al.*, 1988).

In summary, we have studied the ontogeny of the following parameters to elucidate developmental changes in the transmembrane signalling system responsible for the control of cardiac Ca^{2+} current: β -adrenoceptors and muscarinic cholinergic receptors, G_i and G_o proteins, Ca^{2+} channels, and influence of Iso-ACh interactions on $\dot{V}_{\max}$ of slow APs (Kojima *et al.*, 1988; 1990, and the present paper). In general, the transmembrane signalling mechanism related to muscarinic cholinergic responses reached a peak level about one week earlier than did the transmembrane signalling mechanism related to β -adrenergic responses. From the viewpoint of the relationship of developmental time courses and β -adrenergic responses (Figure 7), there is a good parallel relationship among the parameters of G_i proteins, Ca^{2+} channels, and $\dot{V}_{\max}$ of slow APs but not of β -adrenoceptors: these receptors attained a peak level about one week earlier than the others. These findings strongly suggest that the functional coupling between muscarinic cholinergic receptors and Ca^{2+} current is already established when the coupling between β -adrenoceptors and Ca^{2+} current starts to operate. The nature of the neuronal factors which regulate the levels of β -adrenoceptors, muscarinic cholinergic

Figure 7 Developmental changes in β -adrenoceptor-related transmembrane signalling system for Ca^{2+} current in rat hearts. Curves are plotted for [^3H]-dihydroalprenolol (DHA) binding sites (β -adrenoceptors: $\circ$), substrates for cholera toxin (CTX)-catalyzed ADP-ribosylation (G_i protein: $\square$), [^3H]-nitrendipine (NTD) binding sites (Ca^{2+} channels: $\blacksquare$), and isoprenaline (Iso)-induced increase of $\dot{V}_{\max}$ (Ca^{2+} current: $\bullet$), relative to adult levels at 100%. The enclosed symbols ($\odot$, $\square$, $\blacksquare$, $\odot$) on each curve indicate the time when each parameter reaches a (near) maximum level. The data for CTX substrates and for potentiating effect of Iso on Ca^{2+} current are taken from Kojima *et al.* 1988 and 1990, respectively, with permission.

inoceptors, and NTD-sensitive Ca^{2+} channels in cardiac cells remains to be determined.

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