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Development of Pharmacological Sensitivity to Adenosine Analogs in Embryonic Chick Heart: Role of A_1 Adenosine Receptors and Adenylyl Cyclase Inhibition

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SUMMARY

The developing chick heart was employed as a model system to explore temporal correlations between the onset of pharmacological sensitivity to adenosine analogs and the appearance of A_1 adenosine receptors coupled to adenylyl cyclase. A characterization of the developmental profile for adenosine analog-induced negative chronotropic response revealed that isolated atria from 5- and 6-day embryos were unresponsive to adenosine analogs. The onset of pharmacological sensitivity occurred on embryonic day 7, as evidenced by a 27% reduction in atrial beating rate in the presence of 2-chloroadenosine (2-ClA) (30 μM). The sensitivity of embryonic atria to 2-ClA increased continuously from day 7 to day 12 *in ovo*, when the atria became fully responsive to the negative chronotropic effect of this adenosine analog. In order to evaluate whether the developmental increase in pharmacological sensitivity to 2-ClA reflected changes in the number of A_1 adenosine receptors, the ontogenesis of A_1 adenosine receptors was assessed using the antagonist radioligand 8-cyclopentyl-1,3-[^{3}H]dipropylxanthine as a probe. Cardiac membranes from day 5 and day 6 embryos possessed approximately one third of the maximum number of A_1 adenosine receptors expressed at later embryonic ages. Additionally, agonist/[^{3}H]DPCPX competition curves revealed that the high affinity state receptors comprised a larger proportion of the total receptor population in membranes from day 6 as compared with day 12 embryos. These results suggest that there are pharmacologically inactive A_1 receptors in hearts from day 5 and day 6 embryos. The developmental change in A_1 receptor-mediated negative chronotropic response paralleled the increase in [^{3}H]DPCPX binding sites from embryonic day 7 to day 10. Thus, a large fractional occupancy of A_1 adenosine receptors is required to express negative chronotropy during this period of embryonic development. Studies of the sensitivity of adenylyl cyclase to inhibition by cyclopentyladenosine as a function of ontogenesis revealed that cyclopentyladenosine inhibited basal adenylyl cyclase activity to a similar maximal extent from embryonic day 5 through day 16. The efficacy of cyclopentyladenosine as an inhibitor of adenylyl cyclase activity was, therefore, stable during a developmental period when A_1 receptor density increased approximately 3-fold. Hence, only a fraction of the A_1 receptors present during embryogenesis need to be coupled to produce a maximum response with respect to adenylyl cyclase inhibition, which is an indication of the presence of spare receptors. Considered together, these results demonstrate that the development of sensitivity to A_1 adenosine receptor-mediated negative chronotropic response is not paralleled by developmental changes in adenosine agonist inhibition of adenylyl cyclase. Although the negative chronotropic effect of adenosine has been suggested to be mediated by an inhibition of adenylyl cyclase activity, the lack of temporal correlation between A_1 adenosine receptor coupling to adenylyl cyclase and the responsiveness of isolated atria to adenosine analog-induced negative chronotropy argues against this proposal. The appearance of pharmacologically inactive A_1 adenosine receptors on embryonic day 5 and day 6 may indicate that the functional coupling of these recognition sites to K^+ channels via guanine nucleotide-binding protein is inoperative during this developmental period.

Negative chronotropic properties of adenosine were first reported by Drury and Szent-Gyorgyi in 1929 (1). Additional cardiovascular effects of adenosine include negative inotropic and dromotropic activities, as well as vasodilation of the coronary arteries (2–4). These effects are presumably mediated by a complex series of interactions set in motion at the receptor site by agonist binding. Adenosine receptors have been classified into two classes of membrane-bound receptors, A_1 and A_2, as well as an intracellular P site receptor (5, 6). The extracel-

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ABBREVIATIONS: (*R*)-PIA, N^6-(*R*-phenylisopropyl)adenosine; 2-ClA, 2-chloroadenosine; CPA, N^6-cyclopentyladenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; [^{3}H]DPCPX, 8-cyclopentyl-1,3[^{3}H]dipropylxanthine; G protein, GTP-binding protein; G_i, GTP-binding protein that mediates inhibition of adenylyl cyclase; Gpp(NH)p, guanyl-5′-yl-imidodiphosphate; NECA, 5′-*N*-(ethylcarboxamido)adenosine; (*S*)-PIA, N^6-(*S*-phenylisopropyl)adenosine; 8pSPT, 8-(*p*-sulfophenyl)theophylline; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

lular receptors were identified by their effects on adenylate cyclase activity, with A_1 inhibiting and A_2 stimulating enzyme activity. These receptors are also pharmacologically distinct and can be further characterized by the potencies of various adenosine analogs for each of the subtypes. At A_1 adenosine receptors, the rank order potency of adenosine analogs is (*R*)-PIA > NECA > 2-ClA > (*S*)-PIA. In contrast, at A_2 adenosine receptors, the potency series for these agonists is NECA > 2-ClA > (*R*)-PIA > (*S*)-PIA (7–9). The difference in potencies of (*R*)- and (*S*)-PIA for A_1 receptors is greater than that found for A_2 receptors (9). In addition, the extracellular site requires an intact ribose structure for activity and exhibits a high affinity for agonists, whereas the internal P site requires an intact purine ring and exhibits a low affinity for agonists (5). Physiological and pharmacological evidence suggests that the negative chronotropic response to adenosine and adenosine analogs in various species such as the guinea pig, rat, and rabbit is mediated by an interaction with receptors of the A_1 subtype (10–16).

The sequence of molecular changes by which adenosine regulates heart rate have not been defined. Although the pharmacological profile for the negative chronotropic response indicates the involvement of A_1 adenosine receptors, the transmembrane signaling mechanisms that underlie these physiological effects remain unclear. A number of events have been reported to occur in cardiac tissue in response to stimulation by adenosine agonists, such as inhibition of basal and catecholamine-stimulated adenylyl cyclase activity (17, 18), activation of an inwardly rectifying K^+ channel (19, 20) and inhibition of a slow inward Ca^{++} channel (21, 22). In attempts to investigate the role of regulation of adenylyl cyclase activity in adenosine receptor-mediated cardiac responses, some investigators have shown stimulation of adenylyl cyclase (23), whereas others have reported an inhibition (4, 10, 24) or no influence (25). To better define the molecular mechanisms that mediate the negative chronotropic response of adenosine analogs, we have utilized the developing embryonic chick heart, which has been used extensively to study the biochemical events involved in the development and functioning of various neuroreceptors (26). Because gestational age can be determined precisely, key developmental events, such as the onset of pharmacological responsiveness, can be temporally correlated with the appearance of receptors and receptor coupling to second messenger systems. There is little information regarding the embryonic development of the adenosine receptor in any species, although the development of the cardiac muscarinic receptor in embryonic chick heart, which displays many similarities to the A_1 adenosine receptor in its ability to elicit various physiological and biochemical responses, has been well characterized (27–30). We report here the results of our characterization of the developmental profile of the A_1 adenosine receptor-mediated negative chronotropic response in chick atria. We have also explored correlations between the developmental onset of a pharmacological response to adenosine with the appearance of A_1 adenosine receptors and adenosine analog-induced inhibition of adenylyl cyclase.

EXPERIMENTAL PROCEDURES

Materials. White Leghorn chick embryos were obtained locally from the Poultry Science Department, Oregon State University (Corvallis, OR) and were maintained at 39° in a humidified incubator. The embryonic ages were determined by comparison with the description by Hamburger and Hamilton (31). Chemicals were obtained from the following sources: 2-ClA and GTP were purchased from Sigma Chemical Co. (St. Louis, MO); (*R*)- and (*S*)-PIA, NECA, Gpp(NH)p, and adenosine deaminase from Boehringer-Mannheim (Mannheim, West Germany); [^{3}H]DPCPX from Amersham (Chicago, IL); and CPA, 8-*p*SPT, and unlabeled DPCPX from Research Biochemicals Incorporated (Wayland, MA). All other chemicals were of reagent grade.

Atrial beating rate studies. Measurement of atrial beating rate was performed using a modification of a method developed for evaluating the negative chronotropic effects of muscarinic agonists on embryonic chick heart (32). Intact beating hearts were removed from chick embryos and placed in beating rate medium (149 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 10 mM Mg acetate, 10 mM HEPES, 5.5 mM glucose, and 0.4 mM Na_2HPO_4, adjusted to pH 7.4) that was maintained at 37° and aerated with 100% O_2. The great vessels were removed and the atria were separated from the ventricles under a dissecting scope. The atria were pinned to a Sylgard-coated Petri dish containing 10 ml of beating rate medium and maintained at 37° with a circulating water bath. Six to eight atria were prepared and those demonstrating a stable rhythm with a beating rate between 18 and 32 beats/10 sec, after a 30-min equilibration period, were used for each experiment. Preliminary experiments demonstrated that embryonic atria were capable of maintaining a stable rhythm for periods greater than 3 hr. Beating rates were determined visually with a dissecting microscope by counting beats during three 10-sec epochs, 45–60 sec apart. Control rates for atria were determined before the addition of any drugs and in the presence of the appropriate vehicle. Drugs were dissolved in beating rate medium (2-ClA, NECA, and 8-pSPT) or 0.5 mM HCl [(*R*)-PIA and (*S*)-PIA] immediately before each experiment. 2-ClA was the agonist of choice for these pharmacological studies because of its favorable solubility in beating rate medium. CPA, a more selective A_1 adenosine agonist, required ethanol for solubilization, thus limiting somewhat its usefulness in physiological studies (33). The volume of drug solution added during an experiment never exceeded 7% of the total incubation volume. It was determined in preliminary experiments that the effect of adenosine analogs in decreasing beating rate was maximal within 2 min and maintained for at least 8 min following addition of the drug. Hence, there was no evidence for the development of desensitization during this time interval. The incubation medium was mixed for 10 sec after the addition of a drug, and the beating rate was determined starting 2 min after exposure to drug. For each compound examined, the atria were washed three times with fresh medium following the experiment and were allowed to equilibrate. Thirty minutes after the final wash, beating rates were determined and compared with the initial baseline beating rates to establish reversibility of the drug response. Cumulative concentration-response experiments were performed on 6 to 15 atria from embryonic ages 4 through 10, 12, 14, and 16 days *in ovo*. For those experiments in which the effect of 8-*p*SPT was evaluated, the atria were incubated in 1 to 100 μM 8-*p*SPT dissolved in beating rate medium for 30 min before the start of the concentration-response experiment.

Concentration-response data were analyzed by fitting to a four-parameter logistic equation using the iterative public procedure FITFUN on the PROPHET computer system.

The equation used was:

$$E = E_{min} + (E_{max} - E_{min})/(1 + (X/EC_{50})^n$$

where E_{min} = % of control beating rate at the highest concentration of adenosine analog; E_{max} = 100% of control (i.e., in the absence of adenosine analog); X = concentration of adenosine analog; EC_{50} = concentration of adenosine analog producing 50% of maximum decrease in beating rate; and n = slope factor. Data were normalized by reporting as per cent decrease from the basal beating rate. In addition, the pA_2 value for 8-*p*SPT was determined by the method of Arunlakshana and Schild (34).

Tissue preparation. Intact beating hearts were removed from chick

embryos, great vessels were removed, and the hearts were placed in ice-cold 50 mM Tris buffer (pH 7.5 at 20°) for binding assays or 10 mM HEPES (pH 7.5 at 20°) with 5 IU/ml adenosine deaminase for adenylyl cyclase assays. Hearts to be assayed for the presence of adenosine receptors were placed in 30 volumes of 10 mM Tris buffer and 200 mM sucrose and homogenized with 10–12 strokes in a Dounce homogenizer (B pestle). The homogenate was filtered through two layers of gauze and centrifuged at 35,000 × *g* for 10 min at 4°. The supernatant was discarded and the pellet was resuspended in an identical volume of 50 mM Tris buffer using a Dounce homogenizer. This suspension was recentrifuged as above and the resultant pellet was resuspended using a Dounce homogenizer (A pestle), in 30 volumes of 50 mM Tris buffer (pH 7.5) and was incubated at 22° for 30 min with 5 IU/ml adenosine deaminase, to eliminate endogenous adenosine. After this preincubation, the suspension was recentrifuged as above and the supernatant was discarded. The final pellet was resuspended in 15 to 25 volumes (depending on embryonic age) of ice-cold 50 mM Tris buffer, at a protein concentration of 1 to 2 mg/ml, and was kept on ice until used in the radioligand binding assay.

Cardiac membranes used for experiments evaluating agonist binding to A_1 receptors were prepared as described above with the following modifications. The homogenization buffer consisted of 10 mM Tris (pH 7.5) with 10 mM EDTA, whereas the resuspension buffer consisted of 50 mM Tris (pH 7.5) with 1 mM EDTA. After the first centrifugation step, the membranes were incubated at 37° for 30 min in a buffer containing 1 mM EDTA, 100 mM NaCl, 100 μM GTP, and 7.5 IU/ml adenosine deaminase, to promote dissociation and metabolism of membrane-derived adenosine. This preincubation was followed by three centrifugation (35,000 × *g* for 10 min) and resuspension steps. Both membrane preparations yielded similar specific activities for [^{3}H]DPCPX binding sites.

After dissection, hearts to be used for adenylyl cyclase assays were prepared in a manner similar to that for radioligand binding assays, with a few modifications. A 10 mM HEPES buffer (pH 7.5) was used and the homogenate and suspensions were centrifuged for 30 min at 17,000 × *g*. The final pellet was resuspended in 15 to 25 volumes of 10 mM HEPES buffer, containing 0.3 mM dithiothreitol and 7.5 IU/ml adenosine deaminase, at a protein concentration of 400 to 800 μg/ml.

A_1 adenosine receptor binding assay. The specific binding of the A_1-selective ligand [^{3}H]DPCPX (120 Ci/mmol) to cardiac membranes was determined using a previously described rapid filtration assay, with minor modifications (35). Aliquots (175 μl) of the cardiac membrane preparation (200–300 μg of protein) were incubated for 90 min at 22° with 25 μl of [^{3}H]DPCPX, 25 μl of GTP, Gpp(NH)p, or H_2O, and 25 μl of Tris buffer or competing compound, in a total volume of 250 μl. In experiments in which the densities of A_1 receptors as a function of ontogeny were determined, all assay tubes included 100 μM GTP to promote dissociation of membrane-derived adenosine from A_1 receptors. This procedure eliminated the potential confounding influence of developmental differences in the occupancy of A_1 receptors by endogenously released adenosine, thereby ensuring accurate quantitative comparisons of receptor densities as a function of embryonic age.

The equilibrium binding reactions were terminated by the addition of 2 ml of ice-cold Tris buffer, followed by filtration of the assay tube contents over Whatman GF/C filter strips, which had been presoaked in 0.5% polyethyleneimine (Sigma), using a Brandel cell harvester (Model M-24R; Brandel Instruments, Gaithersburg, MD) under vacuum. Filters were then rinsed with four 4-ml washes of ice-cold Tris buffer to remove unbound radioactivity. Filter disks were placed into counting vials to which 3.5 ml of Biocount scintillation cocktail (RPI Corp., Mount Prospect, IL) was added. Filter-bound radioactivity was determined by liquid scintillation counting (Beckman Model LS 6800), at an efficiency of 53%, after a 6-hr extraction at room temperature. The amount of radioligand bound was less than 3% of the total added ligand in all experiments. Specific binding was defined as total binding minus that occurring in the presence of 1 mM theophylline and represented approximately 70% of the total binding at the K_d values for [^{3}H]DPCPX. In saturation binding experiments, [^{3}H]DPCPX was used in 10–12 concentrations, ranging from 0.10 to 4.0 nM. Saturation isotherm data were analyzed using the Lundon-1 iterative curve-fitting program (36). In all equilibrium saturation binding experiments, the [^{3}H]DPCPX isotherms were adequately described by a one-site model.

Adenylyl cyclase assay. Adenylyl cyclase activity in embryonic chick heart membranes was assayed as described by Salomon *et al.* (37), with minor modifications to optimize inhibition of the enzyme. The final incubation mixture (150 μl) contained 50 mM HEPES buffer (pH 7.5), 100 mM NaCl, 10 mM Mg acetate, 50 μM dATP, 100 μM cAMP, 200 μM papaverine, 100 μM GTP, 1.27 mg/ml creatine phosphate, 0.4 mg/ml creatine phosphokinase, 1.3 mg/ml bacitracin, 0.1 mg/ml egg white trypsin inhibitor, 20–40 μg of membrane protein, and 1–2 × 10^6 cpm/assay tube of [α^{32}P]ATP (40 Ci/mmol). Reactions were initiated with the addition of [α^{32}P]ATP. Preliminary experiments showed that these concentrations of Mg^{2+} (data not shown) and GTP allowed optimal A_1 receptor-mediated inhibition of basal adenylyl cyclase activity. [^{3}H]cAMP (approximately 10,000 cpm/assay tube) was used to monitor recovery. All adenosine analogs were dissolved in 1 to 5% ethanol. The resulting final concentrations of ethanol (0.067–0.3%) had no effect on basal activity of the enzyme. Demonstration of inhibition of adenylyl cyclase activity by the muscarinic receptor agonist carbachol was included in each experiment as an indication of the sensitivity of the experimental conditions. Assays were carried out in triplicate for 20 min at 30° and were stopped by the addition of stopping solution (2% sodium lauryl sulfate, 45 mM ATP, 1.4 mM cAMP) and heating in a boiling water bath for 3 min. Separation of cAMP was achieved by sequential chromatography over Dowex A6 50W-X4 (400 mesh; Bio-Rad, Richmond, CA) and neutral alumina (Sigma), and samples were counted after addition of 10 ml of scintillation cocktail. Concentration-response data for adenosine analog-induced inhibition of adenylyl cyclase were analyzed by fitting a four-parameter logistic equation to these data.

Protein determination. Membrane protein content was assayed by the method of Lowry *et al.* (38) after solubilization of the samples in 0.5 N NaOH. Crystalline bovine serum albumin was used as the standard.

Pharmacological response of isolated atria to adenosine analogs. The effect of increasing concentrations of the adenosine analogs (*R*)-PIA, NECA, 2-ClA, and (*S*)-PIA on the spontaneous beating rate of isolated atria from embryos 12 days *in ovo* is shown in Fig. 1. Control beating rates in the presence of the appropriate vehicle for the four groups, in beats/min, were (*R*)-PIA, 147 ± 8; NECA, 153 ± 7; 2-ClA, 129 ± 14; and (*S*)-PIA, 149 ± 11; they did not differ significantly from each other. The maximal effect achieved with all four adenosine analogs was a complete cessation of spontaneous beating. The EC_{50} values

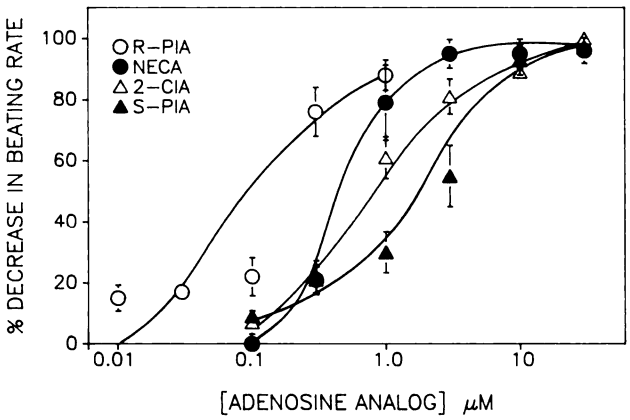

Fig. 1. Concentration-response curves for adenosine analog-induced inhibition of atrial beating rate. The effects of (*R*)-PIA, NECA, 2-ClA, and (*S*)-PIA on the spontaneous beating rates of atria isolated from embryos 12 days *in ovo* were determined. Each value represents the mean ± standard error of 6 to 11 atria. The values are expressed as the mean percentage inhibition of the control beating rate.

derived from analysis of the concentration-response data indicated that the rank order of potency was (*R*)-PIA (0.176 ± 0.065 μM) > NECA (0.516 ± 0.009 μM) > 2-ClA (0.789 ± 0.079 μM) > (*S*)-PIA (2.94 ± 1.74 μM). (*R*)-PIA was approximately 17-fold more potent than (*S*)-PIA at decreasing atrial beating rates. This rank order of potency and stereoselectivity for (*R*)-PIA and (*S*)-PIA is characteristic of a response mediated by an A_1 rather than an A_2 adenosine receptor (6–8).

In order to further verify that this negative chronotropic effect of adenosine analogs was mediated via an extracellular adenosine receptor, the effects of the adenosine receptor antagonist 8-*p*SPT on the response to 2-ClA was investigated. Because most methylxanthines are permeant to cell membranes and exert secondary effects that might modify actions at surface receptors, 8-*p*SPT, a polar methylxanthine, was chosen for these beating rate experiments (13). In the presence of 8-*p*SPT, the 2-ClA concentration-response curve was shifted to the right without a change in slope. This shift in the concentration-response curve is consistent with a competitive antagonism of the response to 2-ClA by 8-*p*SPT (Fig. 2). A Schild plot of these data gave a line with a slope (95% confidence limits) of 0.84 (0.64–1.04) and an apparent dissociation constant for 8-*p*SPT of 0.29 μM.

The development of an adenosine receptor-mediated negative chronotropic response was investigated in 4- through 16-day-old embryos by measuring 2-ClA-induced inhibition of spontaneous beating in isolated atria. Atria isolated from 4-, 5-, and 6-day-old embryos were essentially unresponsive to the negative chronotropic effects of 2-ClA, using concentrations as high as 30 μM. A gradual increase in the maximum negative chronotropic response obtained occurred from day 7 (27% inhibition) to day 9 *in ovo* (45% inhibition), with day 12 atria being fully responsive (100% inhibition) (Fig. 3). This ontogenetic profile was identical to that obtained for (*R*)-PIA-induced inhibition of spontaneous atrial beating (data not shown). A comparison of the EC_{50} values for 2-ClA for all embryonic ages showed that these values were randomly scattered around a mean value of 0.81 μM during development and did not exhibit any trend for an increased or decreased affinity as a function of ontogeny (Table 1). This indicates that, although the maximal response obtained increased with age, the sensitivity of the atria to 2-ClA did not vary significantly.

Ontogenesis of A_1 adenosine receptors. The A_1-selective antagonist radioligand [^{3}H]DPCPX was employed to determine the densities of adenosine receptors during embryonic development. As shown in Fig. 4, [^{3}H]DPCPX binding sites were detectable in 5-day embryonic heart membranes. The number of [^{3}H]DPCPX binding sites increased 2-fold between embryonic day 7 and 9 (29.4 fmol/mg of protein on day 9 *in ovo*) and then remained relatively constant through day 16 *in ovo*.

To determine whether the developmental increase in [^{3}H]DPCPX

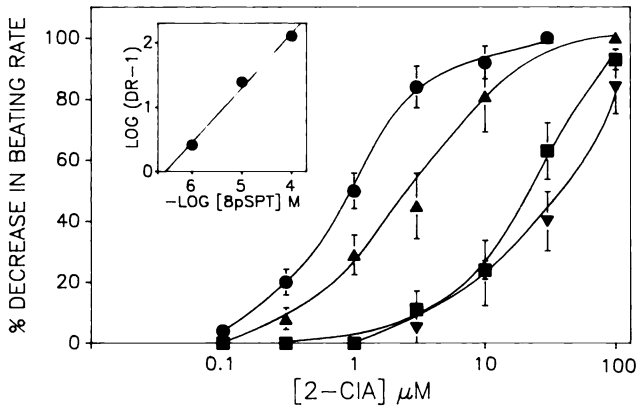

Fig. 2. Antagonism of the 2-ClA-induced inhibition of atrial beating rate by 8-*p*SPT. Spontaneous beating rates were determined in the presence of increasing concentrations of 2-ClA and vehicle (●), 1 μM 8-*p*SPT (▲), 10 μM 8-*p*SPT (■), or 100 μM 8-*p*SPT (▼). Each value represents the mean ± standard error of percentage inhibition of beating rate of five or six atria from embryos 16 days *in ovo*. *Inset*, Schild plot of 8-*p*SPT antagonism data.

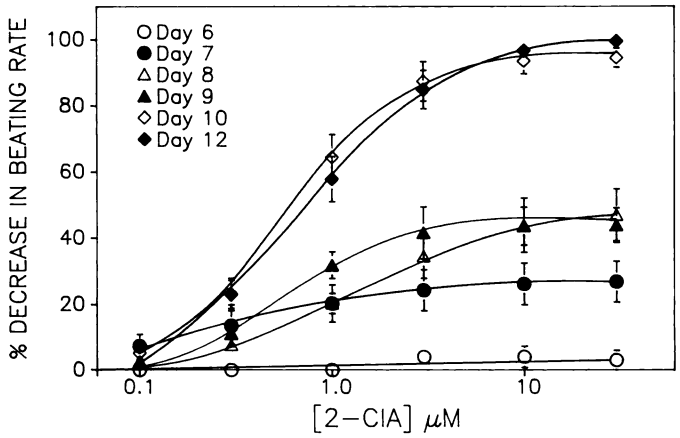

Fig. 3. Concentration-response curves for 2-ClA-induced negative chronotropic effect as a function of embryonic age. Concentration-response data represent the mean ± standard error of percentage inhibition of spontaneous beating rate in atria from embryonic day 6, 7, 8, 9, 10, and 12. Results presented are from 6 to 14 atria for each embryonic age.

TABLE 1

Ontogeny of responsiveness to 2-ClA-induced negative chronotropic response in embryonic chick atria

Concentration-response data were analyzed by fitting a four-parameter logistic equation to these data as described in Experimental Procedures. The values represent the means of 6 to 14 atria for each embryonic age. Numbers in parentheses are 95% confidence limits.

Embryonic age	EC_{50}	Maximum inhibition of atrial beating rate
days in ovo	μM	%
4		4
5		5
6		5
7	0.54 (0.09–0.99)	29.7 (22.5–36.9)
8	1.34 (1.06–1.62)	48.4 (45.6–51.2)
9	0.50 (0.22–0.78)	54.0 (45.8–62.2)
10	0.62 (0.34–0.85)	94.5 (85.0–104.0)
11	0.90 (0.82–0.98)	97.6 (95.0–100.2)
12	0.79 (0.71–0.87)	100.6 (97.5–103.7)
13	1.07 (0.84–1.30)	100.6 (94.4–106.8)
14	0.61 (0.58–0.64)	101.6 (99.8–103.4)
16	0.94 (0.84–1.04)	99.5 (96.7–102.3)

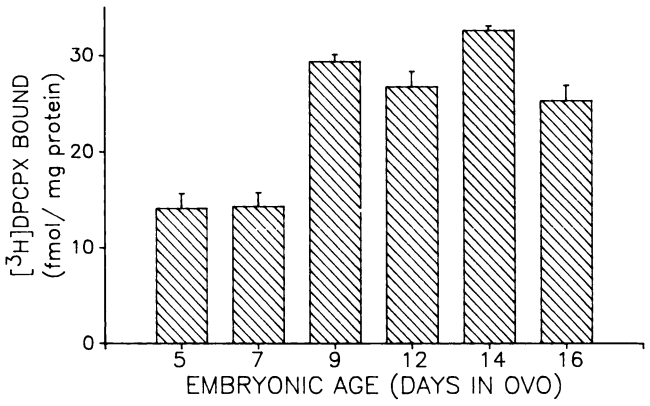

Fig. 4. Developmental profile of [^{3}H]DPCPX binding sites in embryonic chick heart membranes. The results shown are from a representative experiment performed in triplicate, which was repeated twice. The values are expressed as fmol/mg of protein of [^{3}H]DPCPX specifically bound at a single, nonsaturating concentration (1.9 nM) of the radioligand. Specific binding represented an average of 433 cpm. The values for each age are day 5, 14.2 ± 1.6; day 7, 14.5 ± 1.4; day 9, 29.4 ± 0.73; day 12, 26.8 ± 1.6; day 14, 32.8 ± 0.21; and day 16, 25.3 ± 1.6.

binding was related to a change in receptor affinity or density, equilibrium saturation analysis was performed in cardiac membrane preparations derived from embryonic ages 5, 7, 9, and 14 days *in ovo*. The specific binding of [^{3}H]DPCPX was of high affinity, saturable, and to a homogeneous population of recognition sites at all ages investigated. Representative saturation isotherms and corresponding Scatchard replots are shown, for 7- and 14-day embryonic chick hearts, in Fig. 5. The binding parameters derived from nonlinear regression analysis of [^{3}H]DPCPX saturation isotherms, summarized in Table 2, demonstrate that the density of A_1 receptors increased approximately 2.5-fold between embryonic day 5 and day 14. In contrast, the K_d values for [^{3}H]DPCPX binding to A_1 receptors did not change significantly during day 5 through day 14 *in ovo*. Thus, there are no significant developmental changes in A_1 adenosine receptor affinity for [^{3}H]DPCPX during a period when the total number of receptors increases 2.5-fold.

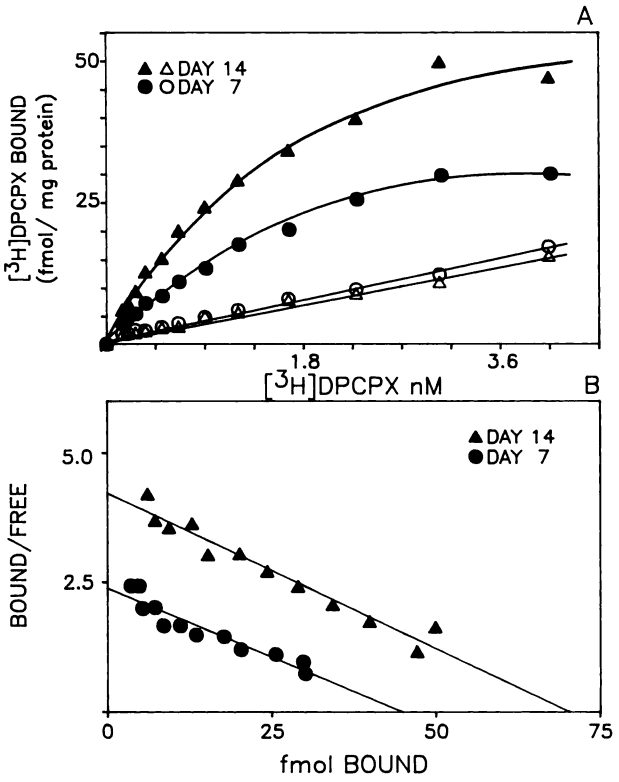

Fig. 5. A, Equilibrium saturation binding of [^{3}H]DPCPX to embryonic chick cardiac membranes. Membranes from embryonic day 7 and day 14 *in ovo* were incubated with 12 concentrations of [^{3}H]DPCPX ranging from 0.1 to 4.0 nM. Values shown are from a single representative experiment, which was replicated twice. The fit shown was obtained using Lundon I saturation analysis software, which yielded a K_d of 2.15 nM and a B_{max} of 48.1 fmol/mg of protein for embryonic day 7 and a K_d of 1.70 nM and a B_{max} of 70.8 fmol/ mg of protein for embryonic day 14. *Closed symbols* indicate specific binding and *open symbols* indicate nonspecific binding. B, Scatchard replot of these saturation data.

TABLE 2

Ontogeny of [^{3}H]DPCPX binding to A_1 adenosine receptors in embryonic chick hearts

The values represent the mean ± standard error of three to six determinations. Individual saturation experiments required 70–80 hearts for embryonic day 5 and 6–8 hearts for embryonic day 14. K_D and B_{max} values were calculated as described under Experimental Procedures.

Embryonic age	K_D	B_{max}
days in ovo	*nM*	*fmol/mg of protein*
5	2.69 ± 0.83	26.9 ± 6.1
7	2.25 ± 0.10	46.8 ± 1.3
9	2.44 ± 0.87	74.8 ± 6.5
14	1.85 ± 0.15	69.2 ± 1.6

Agonist binding to A_1 adenosine receptors. To directly assess the A_1 adenosine receptor-G protein coupling in unresponsive and fully responsive hearts, competition binding experiments were performed, using membranes derived from embryonic day 6 and day 12 hearts. Agonist-antagonist competition curves were carried out in the presence and absence of Gpp(NH)p, using [^{3}H]DPCPX and unlabeled CPA. Adenosine receptor agonists discriminate two affinity states of the A_1 receptor. Hence, computer-assisted analysis of CPA competition data was used to determine the high and low affinity constants (K_H and K_L), as well as the fraction of receptors in each affinity state. These assays were performed in Mg^{2+}-free buffer, to optimize conditions for the detection of alterations in agonist binding resulting from developmental changes in receptor-G protein coupling efficiency (39). Table 3 summarizes the computer-derived binding parameters for CPA/[^{3}H] DPCPX competition curves in embryonic day 6 and day 12 membranes. Under these assay conditions, the distribution of A_1 receptors between high and low affinity forms differed in cardiac membranes of the two embryonic ages. The high affinity state receptors comprised a larger proportion of the total receptor population in membranes from day 6, as compared with day 12, embryos (R_H = 79.2 and 46.8%, respectively). Moreover, in four of the seven experiments performed with cardiac membranes from embryonic day 6, a one-site model with a high affinity state receptor adequately described the competition curves. In contrast, a two-state binding model significantly improved the fit, when compared with the fit of the data to a one-site model, in six of six experiments with embryonic day 12 cardiac membranes. These results suggested a decrease in A_1 receptor-G protein coupling in embryonic day 12 membranes, compared with that observed in embryonic day 6 hearts. To further characterize these differences in the coupling between A_1 adenosine receptors and G proteins at two developmental ages, guanine nucleotide regulation of CPA binding was examined in embryonic day 6 and day 12 membranes. In the presence of 10 μM Gpp(NH)p, the percentage of high affinity sites was significantly ($p < 0.05$) reduced, from 79.2 to 26.5%, in embryonic day 6 membranes (Table 3). In two of three experiments at this embryonic age (day 6), the receptor population manifested only one homogeneous state of low affinity in the presence of Gpp(NH)p. In contrast, CPA binding to embryonic day 12 cardiac membranes was not significantly affected by the presence of 10 μM Gpp(NH)p (Table 3). This relative insensitivity of agonist binding to A_1 receptors of embryonic day 12 membranes is depicted in the representative competition experiments presented in Fig. 6. These results suggest that the increased coupling of A_1 adenosine receptors to G proteins in embryonic day 6 membranes rendered these receptors more sensitive to the Gpp(NH)p-induced conversion to a low affinity state.

Adenosine analog-induced inhibition of adenylyl cyclase activity. CPA-induced inhibition of basal adenylyl cyclase activity was used as a biochemical measure of A_1 adenosine receptor function during embryogenesis. These assays permit an assessment of the extent of coupling of A_1 receptors to adenylyl cyclase through a guanine nucleotide regulatory protein. The highly selective A_1 receptor agonist CPA was used in these studies, to eliminate potential interactions with A_2 adenosine receptors present in the coronary vasculature, which develops during this same embryonic period (33, 40). The dependence of CPA-induced inhibition of adenylyl cyclase activity on GTP concentration is depicted in Fig. 7. CPA did not affect adenylyl cyclase activity in the absence of GTP; however, at GTP concentrations greater than 0.1 μM, the A_1-selective agonist inhibited activity. The EC_{50} for GTP-induced expression of this inhibitory modulation was 2.5 μM (fig. 7, *inset*). In the presence of 100 μM GTP, CPA attenuated basal adenylyl cyclase activity by 16–17% in membranes from 8-day embryonic chick hearts. To further establish the role of A_1 adenosine receptors in the observed GTP-dependent inhibition of adenylyl cyclase, the influence of DPCPX on CPA-induced inhibition of the enzyme was examined. As shown in Fig. 8, DPCPX elicited a concentration-dependent antag-

TABLE 3

Influence of embryonic age on CPA competition for [^{3}H]DPCPX binding to myocardial membranes

Values presented are the mean ± standard error for 3–7 individual experiments. K_H and K_L are, respectively, the high and low affinity equilibrium dissociation constants, whereas % R_H and % R_L represent the percentage of receptors in either the high or low affinity states. The inhibtion constants and percentages of receptors in a given state were determined using the iterative curve-fitting routine LIGAND. In membranes derived from embryonic day 6 hearts, a one-site model of high affinity receptors adequately described the data in four of seven control experiments, whereas a two-site model significantly ($p < 0.01$) improved the fit in six of six control experiments in day 12 membranes. In contrast, in the presence of 10 μM Gpp(NH)p in two of three experiments with embryonic day 6 membranes, the data were adequately described by a one-site model of low affinity receptors. *n*, number of experiments.

Embryonic age	Treatment	*n*	K_H	R_H	K_L	R_L
			nM	%	nM	%
Day 6	Control	7	4.49 ± 1.4	79.2 ± 10.4	115.0 ± 74.9	20.8 ± 10.3
	Gpp(NH)p	3	9.43	26.5 ± 26.5[a]	230.1 ± 103	73.5 ± 23.4[a]
Day 12	Control	6	1.79 ± 0.3	46.8 ± 9.7	230.8 ± 72.8	53.2 ± 9.7
	Gpp(NH)p	3	7.27 ± 1.0	38.5 ± 19.7	158.9 ± 53.8	61.5 ± 19.7

[a] Statistically significant difference from the respective day 6 control value using *t* test for paired data ($p < 0.05$).

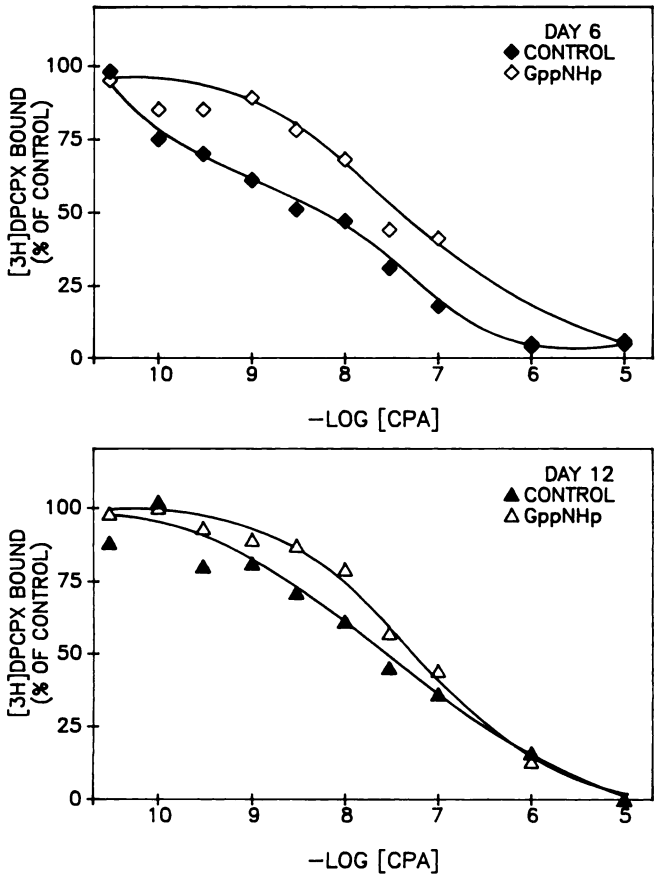

Fig. 6. CPA competition for [^{3}H]DPCPX specific binding to membranes derived from embryonic day 6 or day 12 hearts in the absence (*closed symbols*) and presence (*open symbols*) of 10 μM Gpp(NH)p. Cardiac membranes were incubated with 1.4 nM [^{3}H]DPCPX and increasing concentrations of CPA in this representative experiment. Each experimental condition was repeated 2–6 times with similar results, which are summarized in Table 3. The competition curves were fitted using the iterative public procedure NEWFITSITES2 on the PROPHET computer system, assuming both one- and two-site models. The results presented depict the relative insensitivity of CPA binding to A_1 adenosine receptors of embryonic day 12 as compared with embryonic day 6 membranes to the regulatory influence of 10 μM Gpp(NH)p.

onism of the inhibition of adenylyl cyclase induced by 10 μM CPA, consistent with the involvement of A_1 receptors in the observed inhibitory modulation of the enzyme.

The developmental responsiveness to CPA-induced inhibition of basal adenylyl cyclase activity was investigated in 5- through 10-, 12-, and 16-day embryonic chick cardiac membranes. There were no signif-

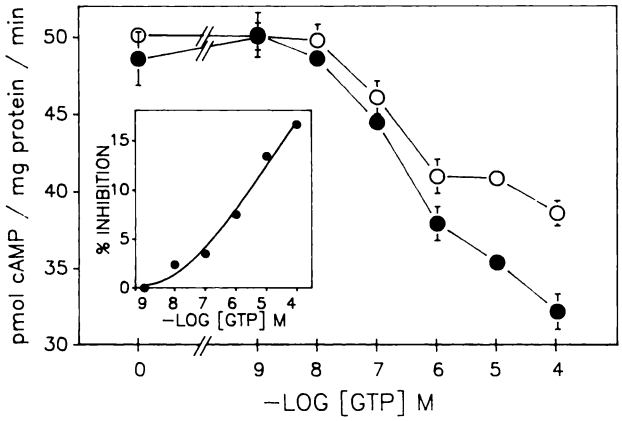

Fig. 7. GTP-dependent inhibition of adenylyl cyclase in embryonic chick heart by CPA. Cardiac membranes prepared from day 8 embryos were assayed for adenylyl cyclase activity with the indicated concentrations of GTP in the presence (●) or absence (○) of 100 μM CPA. *Data points* represent the mean values ± standard errors of triplicate determinations from a single experiment. The experiment was replicated twice with similar results. *Inset*, The per cent inhibition of basal adenylyl cyclase activity produced by 100 μM CPA is plotted as a function of GTP concentration. The maximal per cent inhibition by CPA was 17% in this experiment and occurred at a GTP concentration of 100 μM.

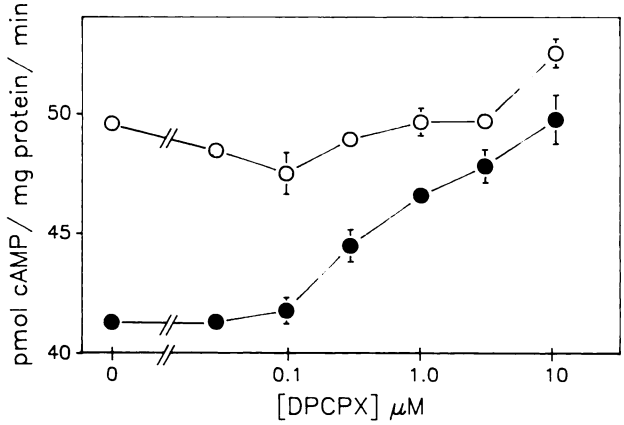

Fig. 8. Effect of DPCPX on the CPA-induced inhibition of basal adenylyl cyclase activity. Cardiac membranes prepared from day 9 embryos were assayed for adenylyl cyclase activity in the presence (●) or absence (○) of 10 μM CPA. *Data points* represent the mean values ± standard errors of triplicate determinations from a single experiment. The experiment was replicated twice with similar results.

icant differences in basal adenylyl cyclase activities observed between days 5 and 16 *in ovo* (Fig. 9). The maximum inhibition of basal adenylyl cyclase activity by CPA was approximately 10 to 15% and was invariant during this period of embryonic development (Fig. 9). Thus, the efficacy of CPA to inhibit basal adenylyl cyclase activity remained constant during a developmental period in which the physiological sensitivity of chick atria to adenosine agonists changed from unresponsive to fully responsive. Representative concentration-response curves for CPA-induced inhibition of adenylyl cyclase activity in 6-, 8-, and 10-, and 16-day embryonic chick heart membranes are depicted in Fig. 10. The IC_{50} values (± SE; n=3 for each embryonic age) for inhibition for adenylyl cyclase by CPA ranged from 0.91 ± 0.28 μM at day 6 to 0.39 ± 0.16 and 0.59 ± 0.10 μM at day 10 and day 16, respectively. Although these mean IC_{50} values were not statistically different, there was a definite trend of increasing potency of CPA as a function of embryonic development. The increased potency of CPA as an inhibitor of adenylyl cyclase is likely to be a consequence of developmental increases in A_1 receptor density and is, therefore, consistent with the existence of spare receptors.

The studies of adenosine receptor-mediated regulation of basal adenylyl cyclase activity, to this point, employed membrane preparations derived from whole embryonic hearts. Therefore, to assess the possibility that sensitivity to CPA inhibition of adenylyl cyclase did not develop coordinately in atria and ventricles, CPA concentration-response curves were performed in atrial membranes from 8-, 10-, 12-, and 16-day embryos. Although the sensitivity of atrial adenylyl cyclase activity to inhibition by CPA tended to be slightly greater than that observed in whole heart homogenates, the developmental profile was similar in both preparations (Fig. 11). Thus, CPA-induced inhibition of basal adenylyl cyclase in both atrial and whole heart homogenates appeared to be relatively stable during this period of embryonic development.

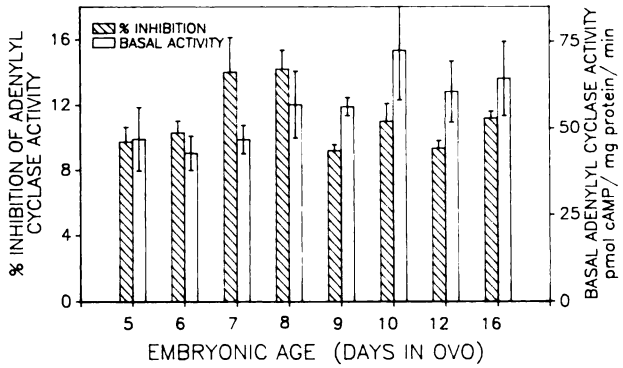

Fig. 9. Developmental profile of basal adenylyl cyclase activity and maximal percentage of inhibition of adenylyl cyclase by CPA in embryonic chick heart membranes. Cardiac membranes prepared from embryos of the ages indicated were assayed for adenylyl cyclase activity in the presence of 100 μM GTP. *Bar heights* represent either the per cent inhibition of basal activity in response to 10 μM CPA or basal adenylyl cyclase activity in pmol of cAMP/mg of protein/min. Each value is the mean ± standard error of two or three separate determinations for each embryonic age.

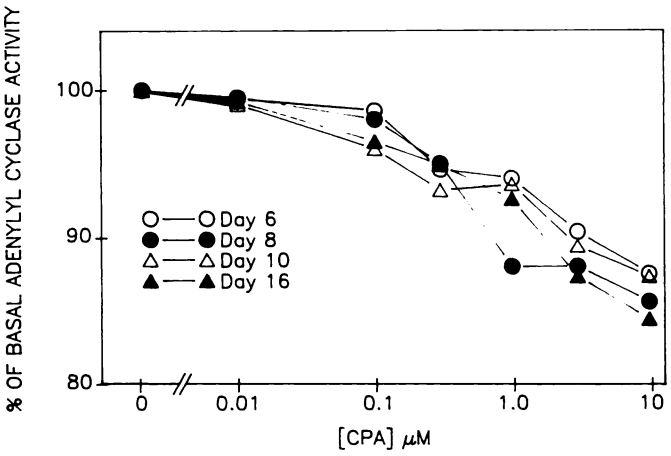

Fig. 10. Concentration-response curves for CPA-induced inhibition of adenylyl cyclase activity in embryonic day 6, 8, 10, and 16 cardiac membranes. Values shown are means of triplicate determinations in a single representative experiment. These experiments were replicated two to four times for each embryonic age, with similar results.

Discussion

The pharmacological profile of the cardioinhibitory effects of adenosine suggests the involvement of A_1 adenosine receptors; however, the relevance of their coupling to adenylyl cyclase remains equivocal (41). In the present study, the developing chick heart has been employed as a model system to explore temporal correlations between the onset of pharmacological sensitivity and the appearance of A_1 adenosine receptors coupled to adenylyl cyclase. A characterization of the developmental profile for adenosine analog-induced negative chronotropic response revealed that isolated atria from 5- and 6-day embryos were unresponsive to adenosine analogs. The onset of pharmacological sensitivity occurred on embryonic day 7, as evidenced by a 27% reduction in atrial beating rate in the presence of 2-ClA (30 μM). The sensitivity of embryonic atria to 2-ClA increased continuously from day 7 to day 12 *in ovo*, when the atria became fully responsive to the negative chronotropic effect of the adenosine analog. The competitive antagonism of the response to 2-ClA by 8pSPT confirmed the receptor-mediated nature of this negative chronotropic effect. Moreover, the rank order potency for the adenosine analogs examined as inhibitors of atrial beating rate was consistent with an A_1 adenosine receptor-mediated response.

In order to evaluate whether the developmental increase in pharmacological sensitivity to 2-ClA reflected changes in the number of A_1 adenosine receptors, the ontogenesis of A_1 adenosine receptors was assessed, using the antagonist radioligand [^{3}H]DPCPX as a probe (35). DPCPX is an adenosine receptor antagonist with a high selectivity for A_1 over A_2 receptor subtypes, as demonstrated by its ability to selectively block the

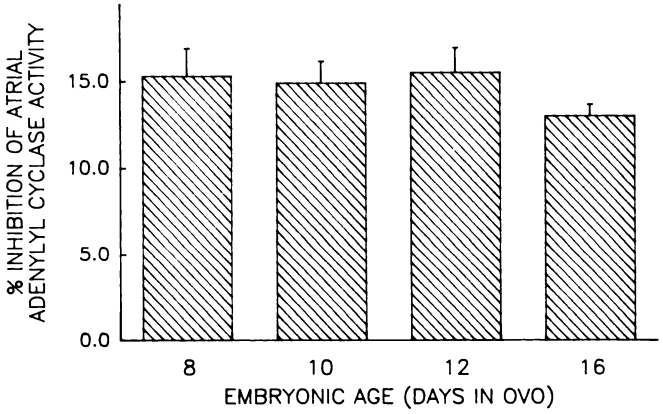

Fig. 11. Developmental profile of maximal per cent inhibition of adenylyl cyclase by CPA in embryonic chick atrial membranes. Atrial membranes prepared from embryos of the ages indicated were assayed for adenylyl cyclase activity in the presence of 100 μM GTP. *Bar heights* represent the per cent inhibition of basal activity in response to 10 μM CPA. Each value is the mean ± standard error of three separate determinations for each embryonic age depicted.

negative chronotropic action and not the coronary vasodilator action of (*R*)-PIA in isolated rat heart (18). The relationship between the developmental profiles for pharmacological sensitivity of the chick atrium to 2-ClA and for A_1 adenosine receptor number is depicted in Fig. 12. These data, normalized to their respective maximum values during embryonic development, indicate that the appearance of A_1 adenosine receptors precedes the onset of pharmacological sensitivity to an adenosine agonist. These results suggest that there are physiologically inactive A_1 adenosine receptors in hearts from day 5 and 6 embryos. The developmental change in A_1 receptor-mediated negative chronotropic response paralleled the increase in [^{3}H]DPCPX binding sites from embryonic day 7 to day 10. Thus, it appears that a large fractional occupancy of A_1 adenosine receptors is required to express negative chronotropy during this period of embryonic development. Additional investigations employing quantitative receptor autoradiographic methods will be required to determine whether the ontogeny of A_1 adenosine receptors in the SA node parallels that in the atria and ventricle.

In studies of the development of chick heart muscarinic receptors, Halvorsen and Nathanson (29) have shown that there is an impairment in the coupling of the muscarinic receptor to a guanine nucleotide regulatory component at embryonic day 4, which is no longer present at the eighth embryonic day. To assess the potential involvement of impaired coupling of A_1 receptors to G proteins in the developmental onset of sensitivity to the adenosine analog-induced negative chronotropy, agonist/antagonist competition curves were generated in membranes derived from unresponsive (day 6) and fully responsive (day 12) hearts. The results of these experiments demonstrated that, in the absence of added guanine nucleotides, the high affinity state receptors comprised a larger percentage of the total receptor population in membranes derived from embryonic day 6 as compared with day 12 hearts. These findings argue against the involvement of a defect in the coupling of A_1 receptors to G proteins in the unresponsiveness of embryonic day 6 atria to the negative chronotropic effects of 2-ClA. The differences in the distribution of A_1 receptors between the high and low affinity forms at embryonic days 6 and 12 suggest that the development of pharmacological sensitivity to adenosine analogs is associated with a reduced coupling between the receptor and G proteins. This uncoupling of the A_1 receptor from G proteins subsequent to the development of pharmacological sensitivity to adenosine agonists may be an expression of a desensitization phenomenon. This process of desensitization is likely to represent an adaptive response subserving the regulation of myocyte sensitivity to the cardioinhibitory effects of adenosine. These results were not unexpected, given the previous reports of adenosine agonist-induced down-regulation of A_1 receptor high affinity sites with concomitant reduction in the levels of G_i in adipocyte membranes (42, 43).

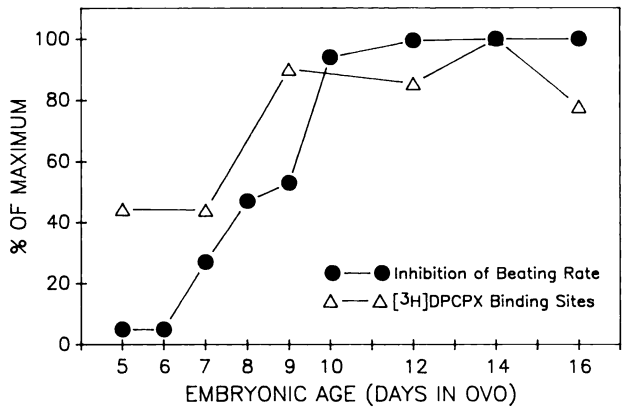

Fig. 12. Relationship between the developmental profiles for the maximal sensitivity to 2-ClA-induced inhibition of atrial beating rate and the number of A_1 adenosine receptors labeled by [^{3}H]DPCPX in embryonic chick heart membranes. Values for 2-ClA-induced suppression of atrial beating rate and [^{3}H]DPCPX binding site number were normalized to the percentage of the maximal value obtained for each parameter. The normalized values for [^{3}H]DPCPX binding sites for each embryonic age were obtained by calculating the percentage of the value obtained on embryonic day 14 (32.8 ± 0.21 fmol/mg of protein), whereas values for sensitivity to the negative chronotropic response to 2-ClA are from the data in Table 1.

The appearance of pharmacologically inactive A_1 adenosine receptors in day 5 and day 6 embryonic chick hearts suggested that a defect in the functional coupling of A_1 receptors to a relevant effector system may underlie the lack of responsiveness of these embryonic ages to 2-ClA. Given the ability of A_1 adenosine receptor activation to affect a G_i-transduced inhibition of adenylyl cyclase, we characterized the sensitivity of adenylyl cyclase to inhibition by CPA as a function of embryogenesis. CPA inhibited basal adenylyl cyclase activity to a similar maximal extent from embryonic day 5 through day 16. Thus, the functional coupling of A_1 adenosine receptors to a GTP-dependent inhibition of adenylyl cyclase was similar in unresponsive and responsive embryonic hearts. The efficacy of CPA as an inhibitor of adenylyl cyclase activity was, therefore, stable during a developmental period when A_1 receptor density increased approximately 2.5-fold. Hence, only a fraction of the A_1 receptors present during embryogenesis need to be coupled to produce a maximum response with respect to adenylyl cyclase inhibition, which is an indication of the presence of spare receptors. Companion experiments have established that carbachol-induced inhibition of basal adenylyl cyclase activity is also invariant between embryonic day 5 and day 16. In accordance with the results of Halvorsen and Nathanson (29), carbachol inhibited basal adenylyl cyclase activity by 24–31% in membranes prepared from embryonic day 5 through day 16 chick hearts (data not shown). Thus, the functional coupling of A_1 adenosine receptors and muscarinic receptors to adenylyl cyclase via G proteins occurs early in embryonic development. However, unlike muscarinic receptor-mediated negative chronotropy, which is nearly fully responsive in embryonic day 5 atria, the physiological sensitivity to A_1 adenosine receptor activation is not maximally expressed until embryonic day 12.

In agreement with the findings of Linden *et al.* (44), we have found that the magnitude of A_1 adenosine receptor-mediated inhibition of adenylyl cyclase is approximately half of that obtained with muscarinic inhibition of the enzyme. This differential efficacy regarding inhibitory modulation of adenylyl cyclase has been attributed to the greater density of muscarinic than A_1 adenosine receptors in rat heart (44). Consonant with this suggestion, the density of A_1 receptors labeled by [^{3}H]DPCPX in the present experiments is approximately 5–10-fold lower than the number of muscarinic receptors previously reported for an embryonic chick heart of an equivalent age (28, 45). The lack of a direct comparison between the receptor occupancy-activity relationships for A_1 adenosine and muscarinic receptors in chick myocytes precludes an evaluation of

the involvement of differences in receptor coupling efficiencies in the observed disparity in the extent of adenylyl cyclase inhibition. However, it may tentatively be proposed that the greater efficacy of muscarinic as opposed to adenosine receptor-mediated inhibition of adenylyl cyclase is a function of both a 5–10-fold higher receptor density and a more efficient coupling of the receptor to this enzyme.

Considered together, these results demonstrate that the development of sensitivity to A_1 adenosine receptor-mediated negative chronotropic response is not paralleled by developmental changes in adenosine agonist inhibition of adenylyl cyclase. Although the negative chronotropic effect of adenosine has been suggested to be mediated by an inhibition of adenylyl cyclase activity (10), the lack of temporal correlation between A_1 adenosine receptor coupling to adenylyl cyclase and the responsiveness of isolated atria to adenosine analog-induced negative chronotropy argues against this proposal. However, we can not exclude the possibility that a step distal to the inhibitory modulation of adenylyl cyclase, such as cyclic AMP-dependent protein kinase or a target protein, does not mature until the developmental stages at which we have demonstrated the appearance of physiological responsiveness. A more plausible molecular mechanism for the A_1 adenosine receptor-mediated negative chronotropic response has been provided by the recent demonstration that adenosine receptors are coupled to K^+ channels via a regulatory G protein in guinea pig atrial tissue (46). Moreover, adenosine and muscarinic receptors have been shown to share the same pool of cardiac K^+ channels in a single cell (46). The changes in physiological sensitivity of the chick atrium to adenosine agonists during embryogenesis may, therefore, be related to the development of functional coupling between A_1 receptors and K^+ channels via regulatory G proteins.

Although a convergence of adenosine receptors and muscarinic receptors on a single pool of K^+ channels has been shown, the ontogenetic profiles for physiological sensitivity to the negative chronotropic effects of muscarinic and adenosine agonists in embryonic chick hearts differ. Whereas isolated chick atria are nearly fully responsive to carbachol-induced inhibition of beating rate by day 5 *in ovo* (29), the A_1 adenosine receptor-mediated negative chronotropic response is not maximally expressed until day 12 *in ovo*. These results suggest that muscarinic and A_1 adenosine receptors may not converge on an identical pool of G proteins and/or K^+ channels in the embryonic chick atria. The appearance of physiologically inactive A_1 adenosine receptors on embryonic day 5 and day 6 may indicate that the functional coupling of these recognition sites to K^+ channels via G proteins is inoperative during this developmental period. Additional investigation of the developing chick heart will be required to more fully define the molecular mechanisms that underlie the A_1 adenosine receptor-mediated negative chronotropic response.

Acknowledgments

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