AN ABSTRACT OF THE THESIS OF

T. Ann Blair for the degree of Doctor of Philosophy in Pharmacy presented on February 9, 1990.

Title: Ontogeny of A_1 Adenosine Receptor-Mediated Negative Chronotropy in Embryonic Chick Heart

Abstract approved: Redacted for privacy
Thomas F. Murray

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 physiological sensitivity occurred on embryonic day 7 and increased continuously to day 12. To evaluate whether the developmental increase in physiological sensitivity 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. Cardiac membranes from day-5 and -6 embryos possessed approximately one-third the maximum number of A_1 adenosine receptors expressed at later embryonic ages. An increase in [^{3}H]DPCPX binding sites occurred at embryonic day-7 and continued through day-10, paralleling a developmental change in the A_1 adenosine receptor mediated negative chronotropic response.

Studies of the sensitivity of adenylyl cyclase to inhibition by cyclopentyladenosine (CPA) as a function of ontogenesis revealed CPA inhibited basal adenylyl cyclase activity to a similar maximal extent

from embryonic day-5 through day-16. The efficacy of CPA as an inhibitor of adenylyl cyclase activity was therefore, stable during a developmental period when A_1 receptor density increased 2.5 fold and physiological sensitivity increased 100 fold. These results demonstrate that the development of sensitivity to the A_1 adenosine receptor-mediated negative chronotropic response is not paralleled by developmental changes in adenosine agonist inhibition of adenylyl cyclase.

In ovo exposure to CPA decreased adenosine receptor density as measured by the specific binding of [^{3}H]DPCPX at both physiologically unresponsive and responsive stages of development. Down regulation of the adenosine receptor was accompanied by a decrease in the ability of CPA to elicit a negative chronotropic response with no significant effect on adenosine receptor mediated reduction in cAMP levels. Agonist/antagonist competition data revealed no measurable effect of down regulation on the relative fractions of receptors in the high and low affinity states or on their respective affinities for CPA. These data suggest chronic exposure to an adenosine analog effects a down regulation of cardiac adenosine receptors that is associated with a diminished physiological resonse to receptor stimulation.

Ontogeny of A_1 Adenosine Receptor-Mediated Negative Chronotropy in Embryonic Chick Heart

by

T. Ann Blair

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed February 9, 1990

Commencement June 1990

APPROVED:

Redacted for privacy

Dr. Thomas F. Murray, Associate Professor of Pharmacy
In charge of major

Redacted for privacy

Dr. Richard A. Ohvall, Dean of the College of Pharmacy

Redacted for privacy

Dr. Thomas J. Maresh, Dean of Graduate School

Date thesis is presented February 9, 1990

Typed by author for T. Ann Blair

ACKNOWLEDGEMENTS

I would like to thank my daughters, Amanda and Amber, for their enthusiastic encouragement and unwavering faith in my endeavors throughout my educational career. My parents have my gratitude for their continuous emotional and financial support.

I also thank all the members of Dr. Murray's laboratory for their encouragement, friendship and helpful advice during my graduate studies. In particular, I would like to thank Drs. Amy Eshleman and Paul Franklin, and Ge Zhang for their support, encouragement, enthusiasm and friendship. I am grateful to Dr. Marco Parenti for his help and kind assistance.

I gratefully acknowledge the financial support of the Oregon Affiliate of the American Heart Association in the form of a Summer Fellowship and Oregon State University Department of Chemistry in the form of Tartar Fellowships.

Finally, I thank Dr. Thomas F. Murray for his invaluable advice, encouragement, patience and financial support. His insightful guidance has provided me with a framework on which to build my scientific career and for this, I am extremely grateful.

Contribution of Co-Authors Chapter 2

All experiments were performed and analyzed by myself under the guidance of Dr. Thomas Murray. Dr. Marco Parenti supplied invaluable expertise in optimizing the adenylyl cyclase experiments.

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CHAPTER 1

INTRODUCTION

This chapter reviews the literature relevant to the research described in subsequent chapters. The research presented in this thesis was directed toward establishing the identity of the adenosine receptor responsible for the adenosine-mediated negative chronotropy present in embryonic chick heart and the developmental profile for this response. Temporal correlation of this event with the appearance of adenosine receptors and adenosine analog induced inhibition of adenylyl cyclase was determined. Research on the regulation of adenosine receptors following in ovo exposure to an adenosine analog was also presented.

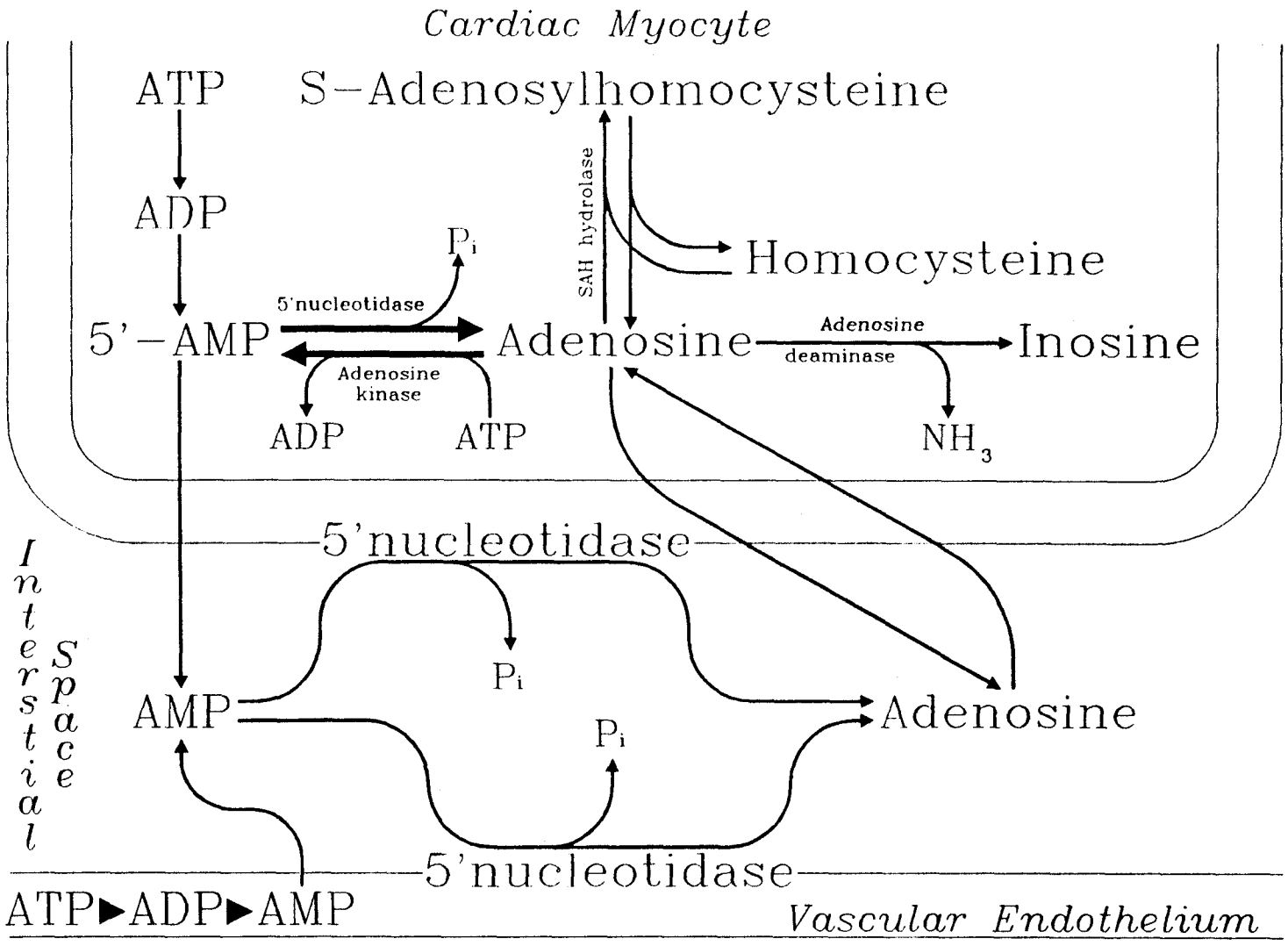
Adenosine has been postulated to play a protective role in the heart (Berne, 1963). During hypoxic or ischemic conditions or as a result of excessive catecholamine stimulation, adenosine formation is increased, leading ultimately to an increase in the coronary blood supply and hence increased O_2supply. A decrease in the work being performed by the heart also occurs in response to increased extracellular adenosine levels. It is the premise of this thesis that through increased knowledge of the cardiac adenosine receptor system gained by studying its development and regulation we increase the potential for development of improved modes of therapy based on this system in the heart. The cardiac adenosine receptor mediated negative

chronotropic response involves a multistep process beginning with the binding of adenosine to a receptor leading to the activation of a transduction mechanism that results in a physiological response. Therefore, in this chapter, a brief discussion of the present state of knowledge regarding each step of this process will be presented beginning first with a description of the formation and metabolism of adenosine followed by descriptions of adenosine receptors and their associated G proteins, effector systems and regulation. The physiological effects of adenosine on the cardiovascular system will be discussed and a short review of the clinical uses of adenosine in cardiovascular therapy will also be presented.

A. Adenosine Metabolism in the Cardiovascular System

Adenosine can be formed both intracellularly and extracellularly by the dephosphorylation of 5'-AMP by 5'-nucleotidase. Adenosine can also be formed intracellularly by the degradation of 5-adenosylhomocysteine by SAH hydrolase (Fig. 1.1) (Schrader, 1983). Although controversy exists over the relative contributions of these enzymes it is generally accepted that under hypoxic conditions, the cytosolic form of 5'-nucleotidase is the enzyme responsible for an increase in adenosine formation (Worku and Newby, 1983, Newby et al. 1987). In addition to hypoxic conditions, elevated adenosine formation via this pathway occurs following such stimuli as ischemia, catecholamines, increased calcium, and sympathetic nerve activation (Berne, 1980; Schrader, 1983). Lloyd and Schrader (1987) have presented data supporting a role for SAH hydrolase as the primary pathway for the

Figure 1.1 Adenosine Metabolism



formation of adenosine under normoxic conditions. Under normal physiological conditions the concentration of intracellular adenosine is maintained at concentrations of 1 1M or lower (Sollevi et al., 1987).

In addition to different enzymes and different subcellular distributions for the enzymes involved in the formation of adenosine, there are also differences in the ability of various cell types to produce adenosine. Cardiac myocytes and cardiac vascular endothelium both produce adenosine and are capable of increasing production in response to hypoxic and ischemic conditions (Sparks and Bardenheuer, 1986). However, Bardenheuer et al.(1987) have recently presented data supporting the role of cardiac myocytes as the primary source of adenosine under conditions in which a modulatory response to increased levels of endogenous adenosine has been detected.

Interstitial and intravascular adenosine is taken up by various cell types including cardiac myocytes, smooth muscle cells, endothelial cells and red blood cells, all differing in their ability to transport adenosine (Rovetta et al., 1987). Adenosine can enter these cells by passive diffusion, facilitated diffusion or sodium-dependent transport (Nees and Gerlach, 1983). Passive diffusion is limited by the hydrophilic nature of adenosine and is only a minor component of adenosine permeation. Facilitated diffusion appears to be the primary mechanism by which the cells of the cardiovascular system transport nucleosides across the membrane and the principle method by which the extracellular actions of adenosine are terminated. This transport system is characterized as a carrier mechanism that is

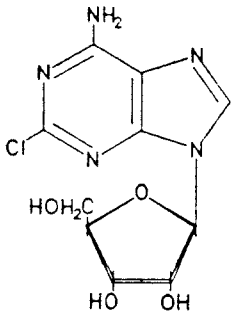
nonconcentrative, exhibits saturation phenomena and is inhibited by specific compounds such as nitrobenzylthioinosine and dipyridamole (Paterson et al., 1983; Gati and Paterson, 1989). Sodium-dependent transport has been identified primarily in renal and intestinal epithelia and therefore does not appear to have a significant role in adenosine metabolism in the cardiovascular system (LeHir and Dubach, 1984).

Intracellular adenosine can be deaminated by adenosine deaminase to inosine or phosphorylated to 5'-AMP by adenosine kinase. The majority of evidence suggests that at low adenosine concentrations, phosphorylation is favored over deamination (supported by low K_m and low V_{max} values for adenosine kinase) while at higher concentrations this enzyme becomes saturated resulting in adenosine deaminase (high K_m and high V_{max} values) being the major pathway for degradation (Arch and Newsholme, 1978).

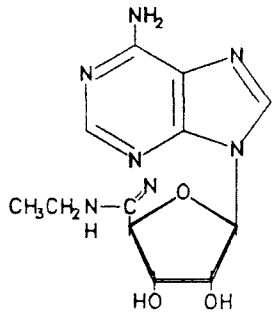
B. Adenosine Receptors

The cardiovascular effects of adenosine were first reported by Drury and Szent-Gyorgyi (1929) and have since been shown to be mediated by a complex series of interactions occurring subsequent to adenosine binding to membrane-bound receptors (James, 1965, Berne, 1963, Schrader et al., 1977). These receptors have been classified into two subtypes of extracellular receptors, A_1 and A_2, and as well as an intracellular P site receptor (Londos and Wolff, 1977). The extracellular receptors were first identified by their effects on adenylyl cyclase activity with A_1 receptors inhibiting and A_2 receptors stimulating activity

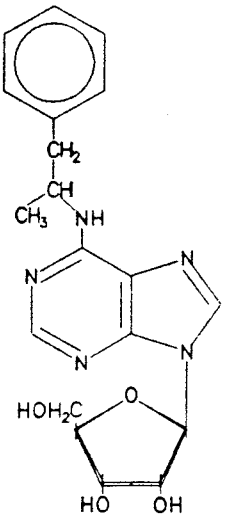
Figure 1.2 Structure of Adenosine Agonists



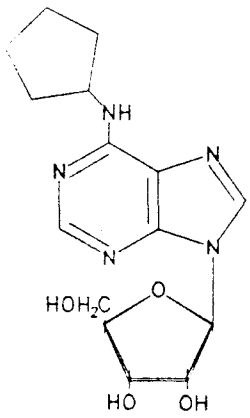
2-Chloroadenosine
(2-CIA)



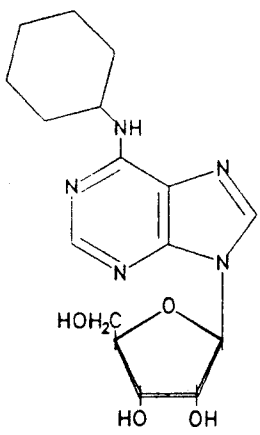
5'-N-Ethylcarboxamidoadenosine
(NECA)



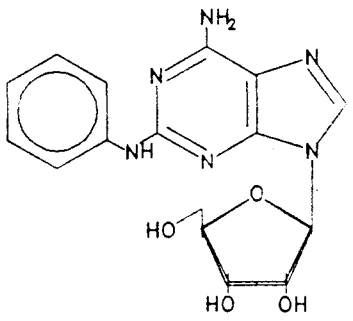
N^6-(2-Phenylisopropyl)adenosine,
R(-) and S(+)-isomer
(R-PIA and S-PIA)



N^6-Cyclopentyladenosine
(CPA)



N^6-Cyclohexyladenosine
(CHA)



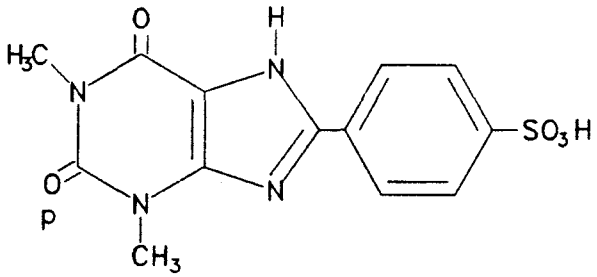
2-Phenylaminoadenosine
(CV-1808)

(Sattin and Rall, 1970, Van Calker et al. 1978, Londos et al, 1980). These receptors are also pharmacologically distinct and can be further characterized by the potencies of various analogs for each of the subtypes (Fig.1.2) (Londos et al, 1980, Schwabe and Trost, 1980). N6-substituted adenosine analogs such as R-phenylisopropyladenosine (R-PIA), cyclohexyladenosine (CHA) and particularly cyclopentyladenosine (CPA) bind with higher affinity to A_1 adenosine receptors than 5'-substituted analogs such as 5'-N-ethylcarboxamidoadenosine (NECA) (Williams et al., 1986). The most selective agonists for the A_2 subtype are the 2'-substituted analogs such as 2-phenylaminoadenosine (CV-1808) with approximately a five fold A_2 selectivity (Bruns et al., 1987) and 2-[*p*-(2-carboxyethyl) phenethylamino]-5'-N-ethylcarboxamidoadenosine (CGS 21680) with approximately a 140-fold A_2 selectivity (Hutchison et al., 1989; Balwierczak et al., 1989). At A_1 adenosine receptors the rank order potency of adenosine analogs is R-phenylisopropyladenosine > 5'-N-ethylcarboxamidoadenosine > 2-chloroadenosine > S-phenylisopropyladenosine. In contrast at A_2 adenosine receptors the potency series for these agonists is 5'-N-ethylcarboxamidoadenosine > 2-chloroadenosine > R-phenylisopropyladenosine $\geq$ S-phenylisopropyladenosine. The stereoselectivity of the different subtypes as demonstrated by the difference in potencies of R- and S-phenylisopropyladenosine is greater for A_1 receptors (10-40 fold) than that found for A_2 receptors (less than 10 fold). Several researchers have reported heterogeneity in the binding of adenosine analogs to A_2 receptors. Daly et al. (1983) have proposed the existence of high affinity A_{2a} and low affinity A_{2b} subclasses with differential

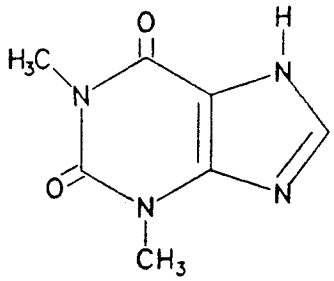
distributions. In addition to the above criteria, it has been demonstrated that the extracellular site requires an intact ribose structure for activity and exhibits a high affinity for agonists while the internal P site requires an intact purine ring and exhibits a low affinity for agonists (Londos and Wolff, 1977). 2',5'-Dideoxyadenosine is the most selective agonist at the P site which is believed to be associated with the enzyme adenylyl cyclase (Daly, 1982; Nimit et al., 1982).

The xanthines currently represent the most potent class of adenosine receptor antagonists (Fig.1.3) (Daly et al., 1987). Methylxanthines such as theophylline and caffeine are competitive antagonists at adenosine receptors and display nearly equal affinity for both the A_1 and A_2 subtypes. Recently, research has led to the development of more potent and subtype-selective antagonists (Martinson et al., 1987). Studies designed to define the structure activity relationships for substituted xanthines as antagonists at the two adenosine receptor-subtypes have shown that antagonist affinity at the A_1 subtype is enhanced by addition of a cycloalkyl moiety to position 8 of the alkylxanthine nucleus. These results led to the development of 8-cyclopentyl-1,3-dipropylxanthine, an adenosine receptor antagonist reported to be 500 to 700-fold more potent at A_1 receptors than at A_2 receptors (Martinson et al., 1987). There are no known high affinity, selective antagonists for A_2 receptors or the P site receptor at present. Alkylxanthines are not antagonists of the internal P-site adenosine receptor.

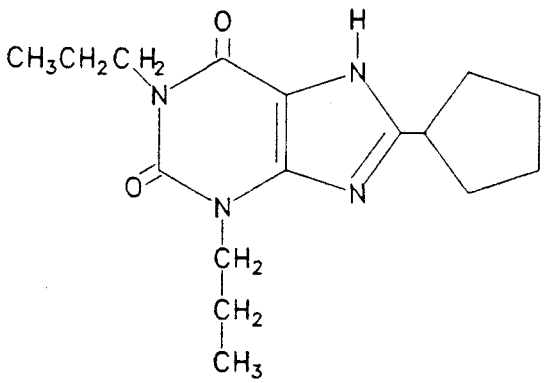
Figure 1.3 Structure of Adenosine Antagonists



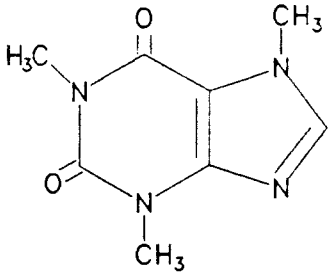
8-(p-Sulfophenyl)theophylline
(8-pSPT)



Theophylline



8-Cyclopentyl-1,3-dipropylxanthine
(DPCPX)



Caffeine

C. Guanine Nucleotide Regulatory Proteins Associated with the Cardiovascular System

Guanine nucleotide regulatory proteins (G proteins) are proteins involved in the transduction of an extracellular signal across the cell membrane to intracellular effector molecules such as adenylyl cyclase. The existence of a protein distinct from the receptor and the enzyme adenylyl cyclase was first hypothesized following the observation that GTP was required for hormonal stimulation of adenylyl cyclase in liver (Rodbell et al., 1971). The existence of G proteins was confirmed in 1981 (Sternweis et al., 1981) by the isolation and purification of a guanine nucleotide regulatory protein. G proteins are heterotrimers composed of a, b and c subunits. Each a-subunit contains a guanine nucleotide binding site with intrinsic ATPase activity and a site sensitive to ADP-ribosylation by cholera and/or pertussis toxin. Originally G proteins were labeled based on their effects on adenylyl cyclase. G_s elicited a stimulatory response, G_i an inhibitory one (Sternweis et al., 1981 and Bokoch et al., 1983). Presently G proteins are named on the basis of the function or suggested function of its a subunit. G_o was the first G protein to be identified whose function was unknown and was thus labeled as the (o)ther G protein (Sternweis et al, 1984). G_t, or transducin, a G protein involved in the activation of cGMP phosphodiesterase in retinal membranes, was identified (Fung et al., 1981) and characterized and G_{olf}, a G protein involved in the transduction of olfactory information, has also been identified (Jones and Reed, 1989). The existence of another G protein, $G_{x/z}$, has recently been proposed that is distinct from other G proteins in that it is not

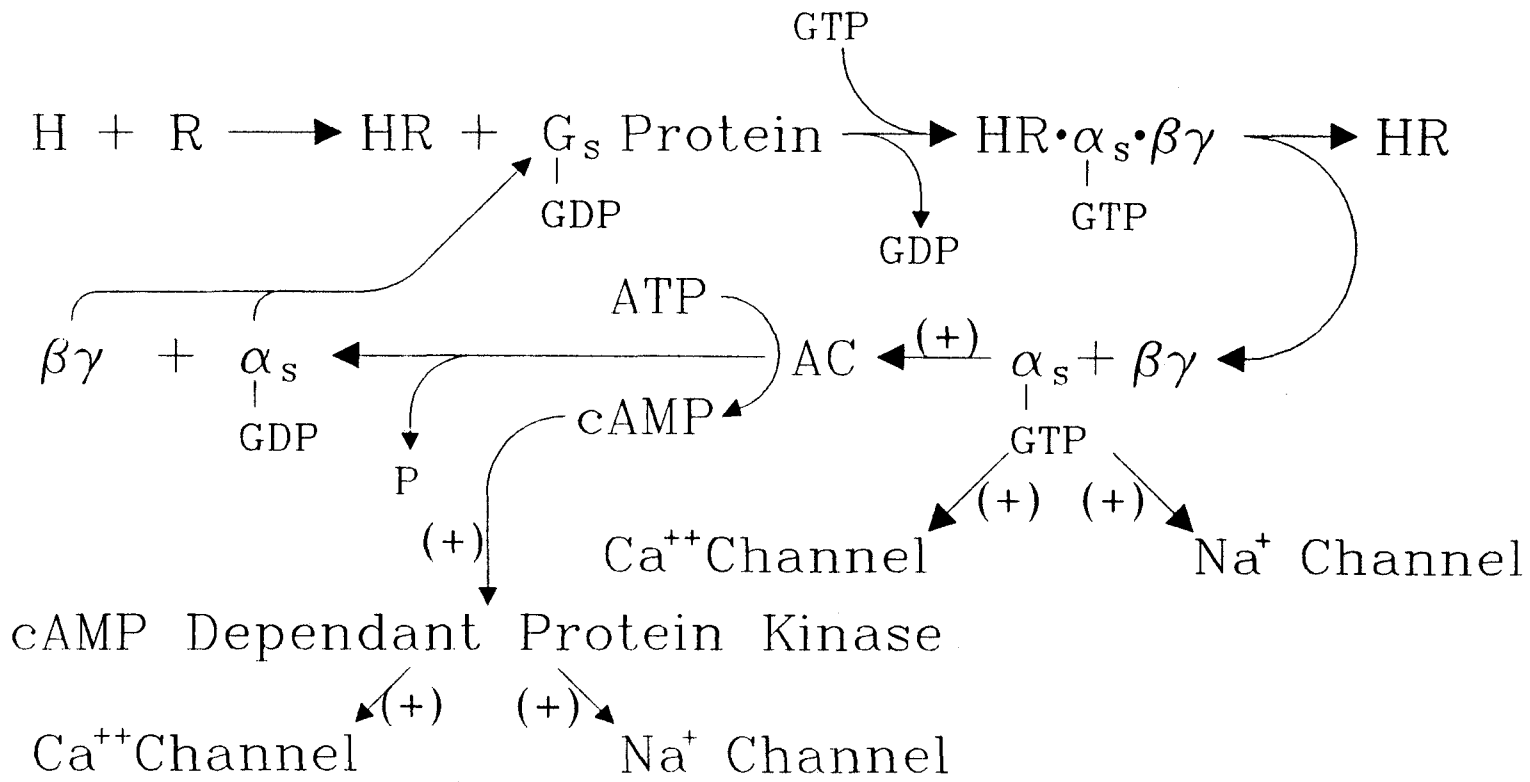
a substrate for either cholera or pertussis toxin and exhibits dramatically lower GTPase activity compared with all other G proteins (Fong and Yoshimoto, 1988 and Matsuoka et al., 1988). The function of $G_{x/z}$ is unknown at this time.

In addition to the different types of G proteins mentioned above, knowledge of the diversity of this system has greatly increased with identification of multiple forms of all three subunits suggesting the possibility of several hundred distinct G protein oligomers. At the present time no unique function has been associated with a specific subunit, however it is postulated that the differences in the subunit structures may be the mechanism by which interactions with different receptors, effector mechanisms and/or regulatory components is accomplished (Gilman, 1987, Mattera et al, 1989, Yatani et al. 1988).

In the cardiovascular system several G proteins have been identified with which adenosine receptors interact either by directly coupling to the G protein or indirectly by G protein-G protein interactions. Support for the role of a G protein in the activation of an effector molecule by adenosine comes from data demonstrating a requirement for GTP, blocking of the response by an ADP-ribosylating toxin, and stimulation of the response in the presence of nonhydrolyzable GTP analogs.

In cardiac tissue $G_{s,}$ coupled to b-adrenergic receptors, is modulated by adenosine in an indirect manner and participates in the mechanism as illustrated in Fig. 1.4. When a hormone (H) binds to the b-receptor (R), G_s-GDP associates with the receptor initiating a conformational alteration of the G protein permitting the dissociation

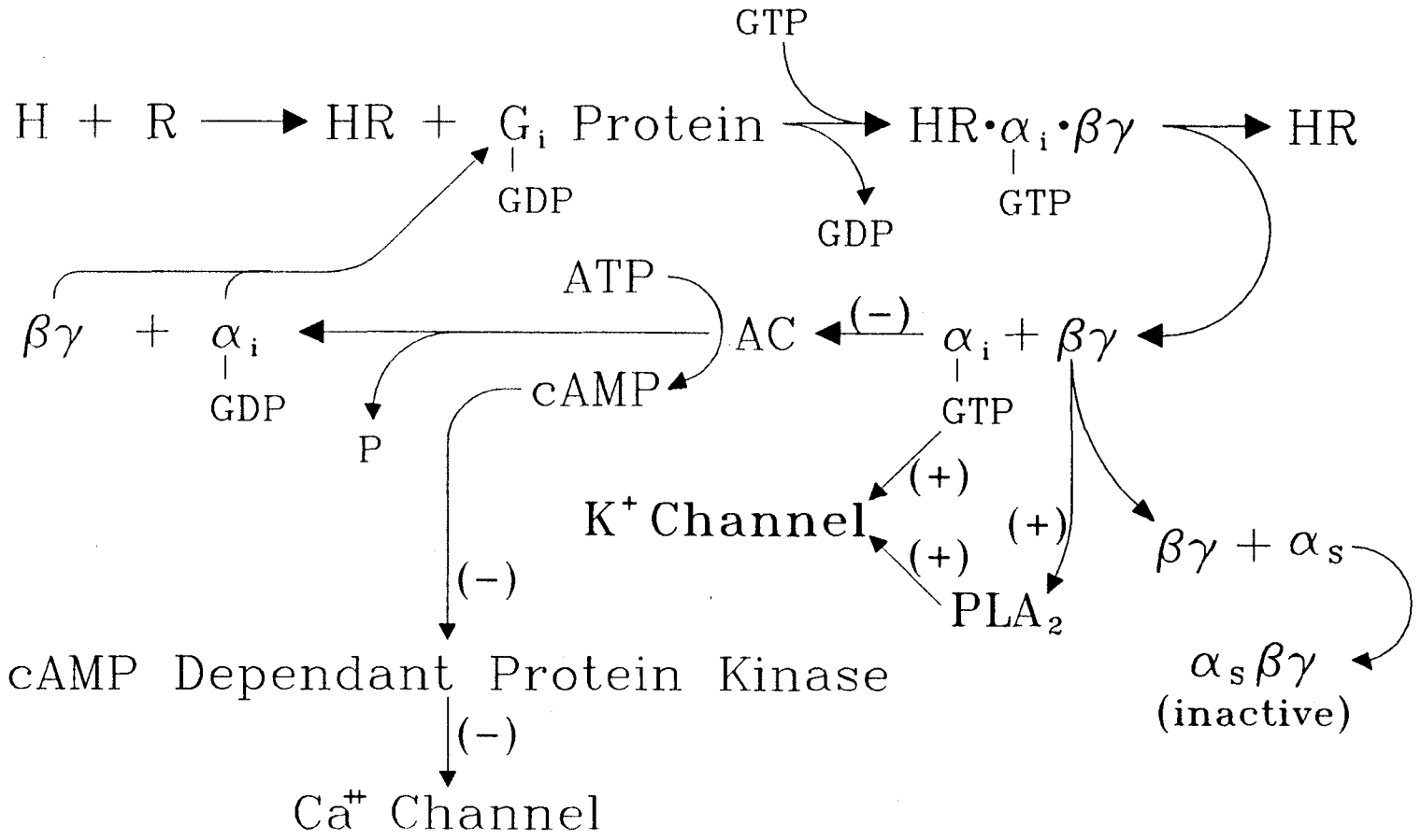
Figure 1.4 Transduction Mechanisms Associated with G_s



of GDP and the association of GTP to the a subunit. This event has two consequences. First, the receptor is converted to a low affinity form causing dissociation of the ternary complex. The receptor can then recycle, activating multiple G proteins during the time that one remains active. This is one level at which amplification of the receptor signal occurs. The second event is the dissociation of the bc subunits from the activated a subunit. The a-GTP subunit in this form is capable of stimulating the activity of adenylyl cyclase (AC) or modulating the activity of other effectors. The a-subunit has intrinsic GTPase activity that eventually results in the hydrolysis of GTP to GDP. However, this occurs relatively slowly resulting in many molecules of cAMP being synthesized per activated a subunit representing another level at which amplification of this system occurs. a-GDP reassociates with the bc subunit to complete the cycle (Gilman, 1989 for review). In vascular tissue, G_s is directly coupled to A_2 adenosine receptors (Anand-Srivastava et al., 1982, Fredholm and Sollevi, 1986). In addition to the activation of adenylyl cyclase, Ingebretsen (1980) suggested that G_s could activate voltage dependent Ca^{++} channels independent of stimulation of adenylyl cyclase activity. Yatani et al. (1987) and Mattera (1989) have presented evidence recently indicating that a_s can directly activate cardiac dihydropyridine-sensitive Ca^{++} channels and Schubert et al. (1989) have presented evidence for a similar effect on voltage-dependent Na^+ channels.

A second G protein identified in the cardiovascular system is G_i which is purported to couple to several cardiovascular receptors including a-adrenergic, muscarinic and adenosine receptors (Gilman,

Figure 1.5 Transduction Mechanisms Associated with G_i



1989 for review). G_i has been linked to both the inhibition of adenylyl cyclase and the activation of a specific K^+ channel with several mechanisms of action proposed for accomplishing both of these effects (Fig. 1.5). Following binding of a hormone to a G_i-coupled receptor, GTP replaces GDP on the a-subunit, resulting in the dissociation of the subunits in a manner similar to what has been described for G_s. The activated a-subunit can then bind to adenylyl cyclase and directly inhibit its activity. However this effect has been reported to be weak compared to the response to a second proposed mechanism. The bc-subunit can bind to the activated a_s-subunit converting it to an inactivated state and thus reducing the activity of adenylyl cyclase in this manner.

The activation of K^+ channels by cardiac muscarinic receptors has also been reported to be accomplished via G_i by two different mechanisms of action. Codina et al. (1987) have reported that a_i-GTP is able to directly activate the channel. A second mechanism proposed by Kim et al. (1989) and Kurachi et al. (1989) involves the activation of phospholipase A_2 (PLA_2) by the bc-subunit with metabolites of this system being the second messengers responsible for the activation of the K^+ channel.

G_o is also found in cardiac tissue and is reported to couple muscarinic cardiac receptors to the metabolism of phosphoinositol (Robishaw and Foster, 1989 for review). In neuronal tissue, adenosine receptors coupled to G proteins have been shown to inhibit inositol-1,4,5-triphosphate (IP_3) turnover in response to histamine receptor stimulation with no direct effect on IP_3 turnover under basal

conditions. Preliminary data in our laboratory has indicated that adenosine may also have a similar function in cardiac tissue in inhibiting muscarinic stimulation of IP_3 hydrolysis (Murray, unpublished observations).

As discussed above G proteins play multiple roles in the regulation of cardiac functioning. Adenosine receptor mediated activation of G proteins fits into this scheme in several areas. First, adenosine receptor-coupled G_i can inhibit the activity of G_s thereby modulating b-adrenergic receptor mediated responses. Inhibition of catecholamine stimulated adenylyl cyclase and subsequent effects by adenosine have been well documented (Dobson, 1978,Hosey et al., 1984, Lohse et al. 1985, Linden et al., 1985,). The ability of adenosine to modulate the cAMP-independent G_s regulation of voltage dependent Na^+ channels and calcium channels has yet to be investigated.

G_i coupled to cardiac A_1 adenosine receptors can directly inhibit adenylyl cyclase and activate K^+ channels (Lohse et al., 1985; Kurachi et al, 1986) and G_s coupled to A_2 receptors in cardiovascular tissue can directly stimulate adenylyl cyclase (Anand-Srivastava, 1982). The possibility of adenosine receptors modulating Ca^{++} channels in a cAMP independent manner via a G protein in a manner analogous to that found in the regulation of K^+ channels has also been proposed although data in support of this hypothesis has been difficult to obtain experimentally.

D. Effector Systems

Adenosine can elicit its cardiac effects via A_1 receptors by both

cyclic AMP-independent (direct) and cyclic AMP-dependent (indirect) mechanisms of action. Direct mechanisms elicit responses following adenosine receptor stimulation in the absence of any prior stimulation of adenylyl cyclase while indirect mechanisms are effective only in conditions that have elevated cAMP levels. In vascular tissue adenosine elicits its response by directly elevating adenylyl cyclase activity or stimulating the release of endothelium derived releasing factor (EDRF) via A_2 receptors. Well established effector systems coupled to adenosine receptors include potassium channels and adenylyl cyclase with proposed effector systems including calcium channels and inositol phosphates (Fig. 1.6 & 1.7).

Direct Effects

In atrial tissue, adenosine receptor stimulation mediates a depressant effect on the SA node (negative chronotropy), a slowing of the AV conduction (negative dromotropy) and a decrease in atrial contractility (negative inotropy). These responses were associated with a hyperpolarization of the cardiac membrane that was first described by Hartzell (1979) in frog sinus venosus following exposure to adenosine. Utilizing electrophysiological experiments and tracer studies with $^{42}K^+$, Jochem and Nawrath (1983) and Belardinelli and Isenberg (1983a) suggested that the hyperpolarization was the result of the activation of a K^+ conductance in guinea pig atria. The adenosine-induced current drives the resting membrane potential toward the K^+ equilibrium potential (E_k) and shortens the action potential. Kurachi et al. (1986) demonstrated that adenosine activates a K^+

Figure 1.6 Functions Associated with A_1 Receptors

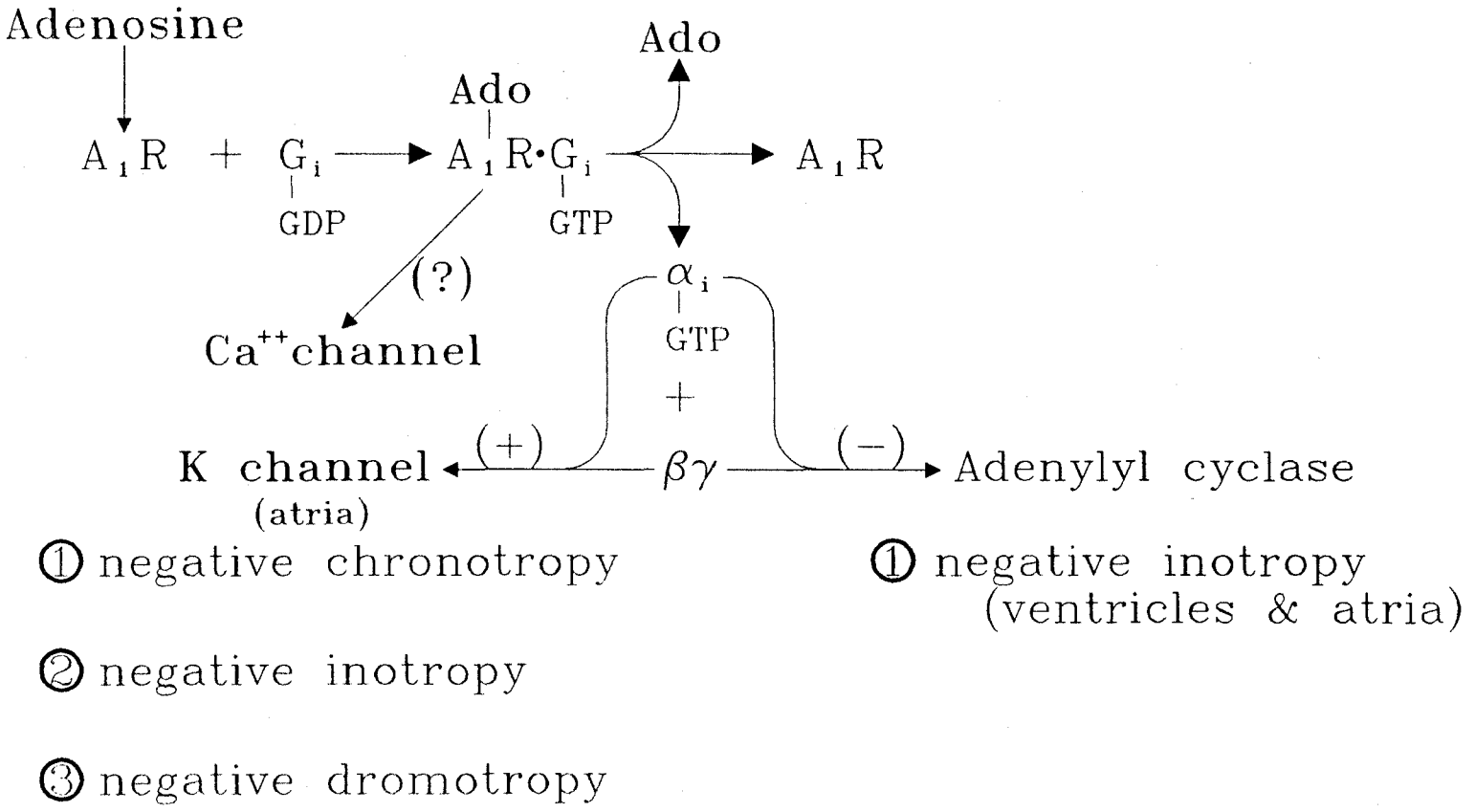
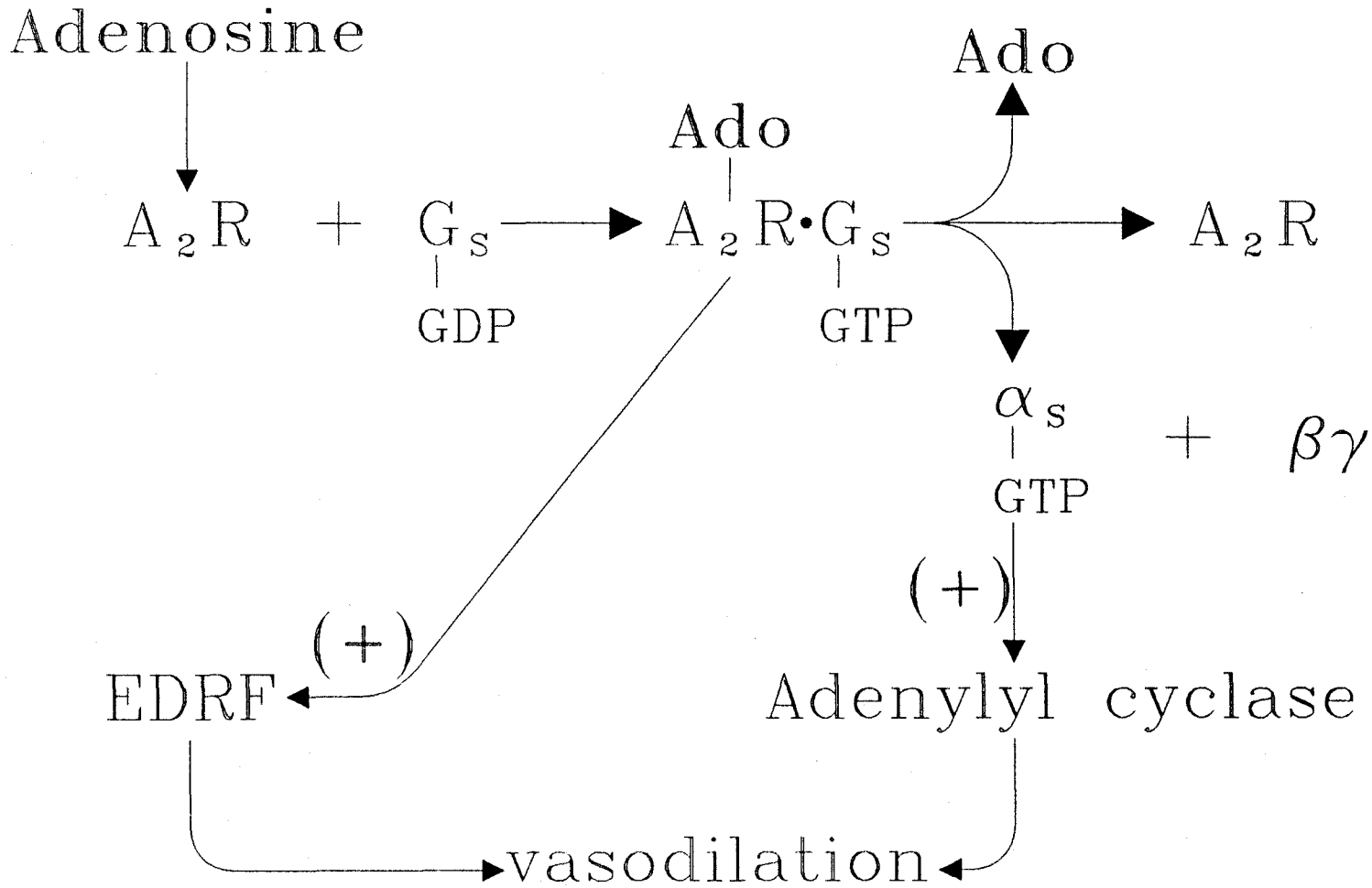


Figure 1.7 Functions Associated with A_2 Receptors



channel in atrial tissue independently of soluble second messengers. This channel appeared to be the same K^+ channel activated by muscarinic receptors. Lewis and Clapham (1989) have recently demonstrated somatostatin receptor mediated activation of the same inwardly rectifying K^+ channel in rat atrial cells. Based on the requirement for GTP, activation of the channel by non-hydrolyzable GTP analogs and pertussis toxin sensitivity (Kurachi et al., 1986; Bohm et al., 1986) it was concluded that the adenosine receptor is linked to the activation of this K^+ channel via a G protein(s), G_i (and/or G_o). As discussed above, recent studies have demonstrated roles for both a_i and bc subunits in the activation of an inwardly rectifying K^+ channel in cardiac tissue that are independent of cAMP concentrations and occur in response to muscarinic receptor stimulation. This suggests the possibility of an analogous mechanism for the activation of K^+ channels in response to adenosine receptor stimulation.

As discussed above A_1 adenosine receptors are coupled to the inhibition of adenylyl cyclase via G_i. Although conflicting results have been reported, the majority of evidence indicates that A_1 receptors in atrial tissue can inhibit basal adenylyl cyclase activity (Anand-Srivastava, 1985; Schrader et al., 1977; Leung et al., 1985; Martens et al., 1987; Bruckner et al., 1985). The function of this decrease in basal activity is unknown although data presented by Linden et al. (1985) indicate a role in modulating contractility in atria.

A_2 adenosine receptors in cardiovascular tissue are coupled to the stimulation of adenylyl cyclase (Anand-Srivastava et al., 1982; Fredholm and Sollevi, 1986). In this tissue adenosine produces

vasodilation in a manner analogous to the mechanism by which stimulation of b-adrenergic receptors produces vasodilation. Cyclic AMP activates a cAMP-dependent protein kinase that is responsible for the phosphorylation of myosin light chain kinase. This process decreases the affinity of myosin light chain kinase for Ca^{++}-calmodulin thus decreasing the phosphorylation of myosin which results in relaxation of vascular tissue (Somylo and Somylo, 1986).

Indirect Effects

Cardiac adenosine receptor stimulation results in the inhibition of adenylyl cyclase activity previously increased by exposure to b-agonists, forskolin and phosphodiesterase inhibitors (Isenberg and Belardinelli, 1984; Hosey et al, 1985). However, adenosine does not antagonize the inotropic and electrophysiological effects of dibutyryl-cAMP or intracellularly applied cAMP. This indicates the site of action for adenosine is at the level of, or proximal to, the formation of the cyclic nucleotide. Adenylyl cyclase is a membrane bound enzyme that catalyzes the conversion of ATP to cAMP. Cyclic AMP is responsible for the activation of a cAMP-dependent protein kinase which phosphorylates a Ca^{++} channel increasing the open channel probability during depolarizing conditions resulting in an influx of Ca^{++} (Sperelakis, 1987). The increase in intracellular Ca^{++} available for contraction is believed to be the mechanism underlying the catecholamine stimulated positive inotropic response . By inhibiting adenylyl cyclase activity, Ca^{++} influx is also inhibited and hence a negative inotropic response occurs following adenosine receptor stimulation.

E. Adenosine Receptor Down Regulation

The regulation of receptors has been extensively studied over the years and although every detail of the biochemical mechanisms involved has not been determined, considerable advances have been made in the past few years. The overall sequence of events involves a series of steps with at least three distinct stages, hormone-receptor binding and desensitization, sequestration and/or internalization of receptors, and loss of receptors (down regulation) (Sibley and Lefkowitz, 1985; Clark, 1986 for review). This working model was developed from early studies investigating the regulation of polypeptide hormones such as epidermal growth factor.

The adenosine receptor system is an example of a macromolecular complex that can be regulated by chronic exposure to an agonist or antagonist. Desensitization of the adenylyl cyclase response and down regulation of the receptor has been demonstrated in fibroblasts and neuronal tissue for A_2 receptors and adipocytes and neuronal tissue for A_1 receptors (Hoffman et al., 1986; Green 1987; Newman and Levitzki, 1983; Lee et al., 1986; Kenimer and Nirenberg, 1981). In addition to the demonstration of desensitization and down regulation of the receptor, Parson and Stiles (1987) and Green (1987) have presented data indicating that down regulation of the A_1 receptor in adipocytes can also be accompanied by a loss of G_i. Shryock et al. (1989) have recently demonstrated a down regulation of the cardiac A_1 receptor following chronic agonist exposure that is accompanied by a decrease in the ability of adenosine to mediate a negative inotropic response. The

effects on inhibition of receptor mediated negative chronotropy and cardiac adenylyl cyclase activity in cardiac tissue has not been evaluated until now.

F. Cardiovascular Responses to Adenosine Receptor Activation

Adenosine has been shown to elicit negative chronotropic, dromotropic and inotropic responses in cardiac tissue and vasodilation of the vasculature.

Negative Chronotropy

The negative chronotropic response to adenosine has been demonstrated both *in vivo* and *in vitro* in a variety of species including rat, guinea pig, rabbit, cat, dog, monkey and human although species variability with regard to certain aspects of their actions has been demonstrated (Belardinelli and Isenberg, 1983b; Samet and Rutledge, 1985; Leung et al., 1985). For example, human and guinea pig AV nodes are more sensitive to the effects of adenosine than the SA nodes and therefore the dromotropic effects of adenosine are more pronounced than adenosine mediated negative chronotropic effects in these species. West et al. demonstrated in whole heart and isolated SA nodal preparations (1985) and isolated SA nodal cells (1987) a dose-dependent decrease in beating rate in response to adenosine. In the intact preparations the slowing was also accompanied by a shift in the leading pacemaker site from the SA node region toward subsidiary pacemaker cells (West and Belardinelli, 1985). In examination of the effects of adenosine on cardiac myocyte action potential parameters,

it was determined that adenosine caused a hyperpolarization of the cell membrane by decreasing the rate of diastolic depolarization (Sperelakis, 1987). The hyperpolarization caused by adenosine correlated well with the degree of sinus slowing. The depressant effects of adenosine on cardiac automaticity were antagonized by adenosine receptor antagonists but not by atropine and potentiated by nucleoside uptake blockers and adenosine deaminase inhibitors (West and Belardinelli, 1985). Neither ouabain or cesium chloride attenuated the hyperpolarization or the sinus slowing indicating adenosine does not elicit its effects by an action on the Na^+K^+ pump or the time and voltage dependent pacemaker current (West et al., 1987). Acetylcholine, via the activation of cardiac muscarinic receptors, also causes hyperpolarization of SA nodal cell through activation of a K^+ channel resulting in a negative chronotropic response. While the mechanism of action for this effect in response to acetylcholine has been well studied, the investigation of the mechanisms of action of adenosine to elicit the same response has just begun. However recent evidence indicates that adenosine and acetylcholine activate the same K^+ channel (Kurachi et al., 1986) and therefore it has been suggested that the activation of an inwardly rectifying K^+ channel, resulting in hyperpolarization of the cardiac membrane is responsible for adenosine's negative chronotropic effect.

Studies with adenosine analogs suggest that the negative chronotropic response to adenosine is mediated by the A_1 receptor subtype (Leung et al., 1985; Samet and Rutledge, 1985). An exception to this is found in the canine heart where the nonselective adenosine

analog NECA is several-fold more potent in eliciting a negative chronotropic response than the A_1 selective agonists R-PIA or CPA (Belloni et al., 1987).

Negative Dromotropy

The dromotropic actions of adenosine as demonstrated by the initiation of heart block following IV administration were first reported in 1929 (Drury and Szent-Gyorgyi, 1929). Similar responses were seen in human heart and have led to adenosine being implicated in mediating atrioventricular conduction disturbances induced by hypoxia (Clemo et al, 1987). In addition to adenosine, adenine nucleotide derivatives (ATP, ADP, and AMP) are also capable of eliciting negative dromotropic responses. However based on the following data, it appears that their actions are dependent on their degradation to adenosine:

1) Methylxanthines antagonize the effects of ATP on AV conduction.
2) Non-hydrolyzable ATP analogs were less potent that ATP.
3) Adenosine transport inhibitors potentiate the effects of ATP
4) Adenosine deaminase attenuated the effects of ATP.
5) ATP, ADP and AMP were all equally potent.

Endothelial cells found in the vasculature have the necessary enzymes to dephosphorylate these compounds to adenosine (Belardinelli et al., 1984).

The site of action for adenosine in mediating a negative dromotropic effect appears to be the AV node. Belardinelli et at. (1980) demonstrated that the conduction velocity between the atrial and the ventricles is slowed in such a way that the atrium to His bundle

interval is increased with no apparent effect on conduction through the bundle of His or ventricular tissue. Clemo and Belardinelli (1986) studied action potential parameters of AV nodal cells and found that adenosine caused decreases in the amplitude, duration and rate of rise of the action potentials with no effect on action potentials of nodal-His, His bundle or ventricular cells.

The negative dromotropic actions of adenosine appear to be mediated by extracellular adenosine receptors of the A_1 subtype. The evidence for this includes competitive antagonism by methylxanthines, potentiation of adenosine's effect by nucleoside transport inhibitors, prolongation of the AV node conduction time by adenosine analogs follows the rank order potency for the A_1 subtype and the inability of the intracellular P-site adenosine analog 2',5'-dideoxyadenosine to elicit a negative dromotropic response in isolated guinea pig heart (Belardinelli et al., 1982; Clemo and Belardinelli, 1986). The mechanism by which adenosine receptors mediate a negative dromotropic response remains to be elucidated. However, Bohm et al. (1989) have provided evidence showing the involvement of a pertussis toxin sensitive G protein in this adenosine mediated effect and, since adenosine has been shown to activate a K^+ channel in atrial cells via a G protein, it is generally thought that this mechanism may also be active here. In addition, muscarinic receptor stimulation, which elicits negative dromotropic responses similar to those mediated by adenosine, does so by the activation of an inwardly rectifying K^+ channel.

Negative Inotropy

Adenosine receptor stimulation elicits negative inotropic responses in both atrial and ventricular myocardium. In atrial tissue adenosine produces two effects, one of which is a direct negative inotropic response that is not associated with a decrease in cAMP levels but is reported to be due to changes in potassium conductance (Belardinelli and Isenberg, 1983a). A receptor mediated increase in K^+ efflux results in shorter action potentials with a decrease in the inward Ca^{++} slow current of the plateau phase. The decrease in Ca^{++} influx decreases the calcium available for contraction and thus results in a negative inotropic response. Adenosine has no effect on action potential parameters or on basal contractility in ventricular myocytes.

Following positive inotropic stimulation adenosine elicits an indirect effect by decreasing adenylyl cyclase activity in both atrial and ventricular tissue. Catecholamines or forskolin, by stimulating adenylyl cyclase activity, increase cAMP levels. As described previously, elevated cAMP levels stimulate cAMP-dependent protein kinases that lead to the phosphorylation of Ca++ channel proteins, an increase in Ca++ influx with a subsequent increase in contractility. Adenosine attenuates this response by decreasing the levels of cAMP (Sperelakis, 1987). Both indirect and direct effects have been shown to be mediated via A_1 adenosine receptors, blocked by methylxanthines and potentiated by procedures that increase the levels of adenosine at the receptor site.

Vasodilation

Adenosine receptors present in the vasculature are capable of eliciting vasodilation in all tissues except in the kidney and in the hepatic venous system where vasoconstriction occurs. Dilation of the coronary vasculature by adenosine was first recognized by Drury and Szent-Gyorgyi in 1929 and has been studied extensively since that time. Their observation was followed by the hypothesis that adenosine acts as a physiological regulator of coronary blood flow in response to hypoxia, ischemia and/or excessive catecholamine stimulation (Berne, 1963). This response is potentiated by adenosine deaminase inhibitors, uptake blockers and antagonized by methylxanthines. The vasodilatory effect of exogenous adenosine has been demonstrated in both *in vivo* and *in vitro* preparations, however, because of the efficient uptake systems in coronary endothelial cells and myocytes, correlations between the concentration of adenosine needed to elicit this response with the actual concentration of adenosine at the receptor site *in vivo* has been difficult to define.

Data derived from binding studies and the rank order potency of adenosine analogs for eliciting vasodilation indicate that the adenosine receptor involved is of the A_2 subtype (Kusachi et al., 1983; Nees et al., 1985). A_2 adenosine receptors are coupled to the stimulation of adenylyl cyclase and produce vasodilation by increasing adenylyl cyclase activity as previously described. An alternative or additional mechanism of adenosine mediated vasodilation has been proposed in a recent study by Kurtz (1987) in which he has presented data indicating the involvement of A_1 receptors located on the vascular

smooth muscle cells of the aorta coupled to the stimulation of guanylyl cyclase activity. Cyclic GMP is a second messenger known to mediate vasodilation in response to nitrocompounds and atrial natriuretic peptide raising the possibility that adenosine may be functioning in a similar manner.

A role for endothelial derived relaxing factor (EDRF) in mediating the vasodilatory effect in response to adenosine has also been proposed (Gordon and Martin, 1983; Frank and Bevan, 1983; Yen et al., 1988). Depending on the species and tissue involved, endothelium-dependent vasodilation mediated by adenosine receptors has been demonstrated. However, the majority of evidence indicates that vascular smooth muscle responds to adenosine in the absence of endothelium.

Another mechanism by which adenosine in known to elicit a vasodilatory effect is by the inhibition of transmitter release from sympathetic nerves responsible for vascular tone via α-adrenergic receptors. This effect is mediated by A_1 adenosine receptors located presynaptically coupled to the inhibition of adenylyl cyclase (Fredholm and Sollevi, 1986).

G. Clinical Uses of Adenosine

A considerable amount of research supports the role of adenosine as a physiologically relevant modulator of the cardiovascular system thus suggesting its use as a therapeutic agent. Clinically, adenosine has been used as an antiarrhythmic agent, for its antiaggregatory properties on platelets and as a vasodilator. A marked advantage of adenosine over many other cardiovascular agents is that its effects are

easily controlled due to the extremely short plasma half life (<1 sec) (Sollevi, 1986).

Adenosine has been used effectively as an antiarrhythmic agent for the termination of paroxysmal supraventricular tachycardia (PVST) in which the AV node is part of the reentry pathway (Belhassen and Pelleg, 1984; Favale et al., 1985). Belardinelli and Isenberg (1983b) have also demonstrated that adenosine is effective in terminating catecholamine-induced ventricular tachycardia. Adenosine has been used as a diagnostic tool for the diagnosis of "sick sinus syndrome" which may be due to a hypersensitivity of nodal tissue to the cardiodepressant actions of adenosine. Tachyarrhythmias such as atrial flutter can also be evaluated more effectively following the induction of AV block with adenosine (Benedini et al., 1984).

An antiaggregatory effect on platelets was first demonstrated by Born and Cross (1963) and is associated with an increase in cAMP mediated by A_2 receptors. Preservation of platelets has been accomplished by the use of adenosine during surgeries requiring extracorporeal circulation systems and in patients with artificial cardiac devices.

The vasodilatory effects of infused adenosine have been utilized in several situations such as the induction of hypotension during surgery to facilitate a variety of surgical procedures. Adenosine is also used for blood pressure control in response to anesthesia-induced hypertension. Vasospasm of the coronary vasculature has been successfully treated with intraarterial infusion of low doses of adenosine. A distinct advantage of adenosine as a vasodilatory agent

in these circumstances is the minimal amount of rebound increased heart rate and hypertension that often occurs with other compounds such as nitroglycerin and nitroprusside (Sollevi et al., 1984).

Acknowledgements

The author gratefully acknowledges the assistance of David Sato with the figures for this chapter.

CHAPTER 2

Development of Pharmacological Sensitivity to Adenosine Analogs in Embryonic Chick Heart: Role of A1 Adenosine Receptors and Adenylyl Cyclase Inhibition

T. Ann Blair, Marco Parenti and Thomas F. Murray

A. 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 pharmacologic sensitivity occurred on embryonic day 7 as evidenced by a 27% reduction in atrial beating rate in the presence of 2-ClA (30 1M). 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 [^{3}H]DPCPX as a probe. Cardiac membranes from day-5 and -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 to day 12 embryos. These results suggest that there are pharmacologically inactive A_1 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, 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 -6 may indicate that the functional coupling of these recognition sites to K^+ channels via guanine nucleotide binding proteins is inoperative during this developmental period.

B. Introduction

Negative chronotropic properties of adenosine were first reported by Drury and Szent-Gyorgy in 1929. Additional cardiovascular effects of adenosine include negative inotropic and dromotropic activities as well as vasodilation of the coronary arteries (James, 1965; Berne, 1963; Schrader et al., 1977). 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 (Londos and Wolff, 1977; Londos et al., 1980). The extracellular 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 (Daly, 1983; Wolff et al.,

1981; Schwabe and Trost, 1980). The difference in potencies of R- and S-PIA for A_1 receptors is greater than that found for A_2 receptors (Schwabe and Trost, 1980). In addition, the extracellular site requires an intact ribose structure for activity and exhibits a high affinity for agonists while the internal P site requires an intact purine ring and exhibits a low affinity for agonists (Londos and Wolff, 1977). 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 (Leung et al., 1985; Samet and Rutledge, 1985; Evoniuk, et al., 1987; Heller and Olsson, 1985; Hamilton et al., 1987; Haleen and Evans, 1985; Haleen et al., 1987).

The sequence of molecular changes by which adenosine regulates heart rate have not been defined. While the pharmacologic profile for the negative chronotropic response indicates the involvement of A_1 adenosine receptors, the transmembrane signaling mechanisms which 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 (Schutz et al., 1986; Hosey, et al., 1984), activation of an inwardly rectifying K^+ channel (Jochem and Nawrath, 1983; Belardinelli and Isenberg, 1983) and inhibition of a slow inward Ca^{++} channel (Caparrotta et al., 1987; Isenberg and Belardinelli, 1983). 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 (Anan-Srivastava, 1985) whereas others have reported an inhibition (Schrader et al. 1977, Leung et al., 1985; Martens et al., 1987) or no influence (Bruckner et al., 1985). To better define the molecular mechanisms which 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 (Pappano, 1977). 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 (Pappano and Skowronek, 1974; Liang et al., 1986, Halvorsen and Nathanson, 1984; Galper et al., 1984). 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 pharmacologic response to adenosine with the appearance of A_1 adenosine receptors and adenosine analog-induced inhibition of adenylyl cyclase.

C. Methods

Materials

White Leghorn chick embryos were obtained locally from the Poultry Science Dept., Oregon State University (Corvallis, OR) and maintained at 39#C in a humidified incubator. The embryonic ages were determined by comparison with the description of Hamburger and Hamilton (31). Chemicals were obtained from the following sources: 2-ClA and GTP were purchased from Sigma Chemical (St. Louis, MO); R- and S-PIA, NECA, GppNHp, and adenosine deaminase from Boehringer-Mannheim (Mannheim, West Germany); [^{3}H]DPCPX from Amersham (Chicago, IL); and CPA, 8-pSPT 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 (Halvorsen and Nathanson, 1981). Intact beating hearts were removed from chick embryos and placed in beating rate media (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 $NaPO_4$ adjusted to pH 7.4) maintained at 37#C and aerated with 100% O_2. The great vessels were removed and the atria separated from the ventricles under a dissecting microscope. The atria were pinned to a Sylgard-coated petri dish containing 10 ml beating rate media and maintained at 37#C with a circulating water bath. Six to eight atria were prepared and those demonstrating a stable rhythm (over 90% of the

atria examined) with a beating rate between 18 and 32 beats/10 seconds 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 three hours. Beating rates were determined visually with a dissecting microscope by counting beats during three 10-second intervals 45-60 seconds apart. Control rates for atria were determined prior to the addition of any drugs and in the presence of the appropriate vehicle. Drugs were dissolved in beating rate media (2-ClA, NECA, 8-pSPT) or 0.5 mM HCl (R-PIA, S-PIA) immediately prior to 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 its usefulness somewhat in physiological studies (Martens, et al., 1987). 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 seconds after the addition of a drug, and the beating rate determined starting 2 minutes after exposure to drug. For each compound examined the atria were washed three times with fresh media following the experiment and allowed to equilibrate. Thirty minutes after the final wash, beating rates were determined and compared to the initial baseline beating

rates to establish reversibility of the drug response. Cumulative concentration response experiments were performed on 6 to 15 atria from embryos at ages 4 through 10, 12-, 14-, and 16-days *in ovo*. For those experiments in which the effect of 8-pSPT was evaluated, the atria were incubated in 1 to 100 1M 8-pSPT dissolved in beating rate media for 30 min prior to 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:

$$E = Emin + (Emax-Emin)/(1 + (X/EC_{50})^n$$

where Emin = % of control beating rate at highest concentration of adenosine analog.

Emax = 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

n = slope

Data were normalized by reporting as % decrease from the basal beating rate. In addition the pA_2 value for 8-pSPT was determined by the method of Arunlakshana and Schild (1959).

Tissue Preparation

Intact beating hearts were removed from chick embryos, great vessels removed, and the hearts placed in ice cold 50 mM Tris buffer

(pH 7.5 at 20#C) for binding assays or 10 mM Hepes (pH 7.5 at 20#C) 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 2 layers of gauze and centrifuged at 35,000 x g for 10 min at 4#C. The supernatant was discarded and the pellet resuspended in an identical volume of 50 mM Tris buffer using a Dounce homogenizer. This suspension was recentrifuged as above, the resultant pellet resuspended using a Dounce homogenizer (A pestle) in 30 volumes 50 mM Tris buffer (pH 7.5) and incubated at 22#C for 30 min with 5 IU/ml adenosine deaminase to eliminate endogenous adenosine. Following this pre-incubation the suspension was recentrifuged as above and the supernatant 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 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, while the resuspension buffer consisted of 50 mM Tris (pH 7.5) with 1 mM EDTA. Following the first centrifugation step the membranes were incubated at 37#C for 30 min in a buffer containing 1 mM EDTA, 100 mM NaCl, 100 1M GTP and 7.5 IU/ml adenosine deaminase to promote dissociation and metabolism of membrane derived adenosine. This preincubation was followed by 3 centrifugation (35,000 x g for 10

min) and resuspension steps. Both membrane preparations yielded similar specific activities for [^{3}H]DPCPX binding sites.

Following 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 x 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 lg/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 (Leid, et al., 1988). Aliquots (175 ll) of the cardiac membrane preparation (200-300 lg of protein) were incubated for 90 min at 22#C with 25 ll of [^{3}H]DPCPX, 25 ll of GTP, GppNHp or H_2O, and 25 ll Tris buffer or competing compound in a total volume of 250 ll. In experiments in which the densities of A_1 receptors as a function of ontogeny were determined, all assay tubes included 100 lM 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 x 4-ml washes of ice-cold Tris buffer to remove unbound radioactivity. Filter disks were placed into counting vials to which 3.5 ml Biocount Scintillation cocktail (RPI Corp., Mount Prospect, Ill) was added. Filter-bound radioactivity was determined by liquid scintillation spectrometry (Beckman model LS 6800) at an efficiency of 53% following a 6 hour 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 Kd 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 (Lundeen and Gordon, 1985). 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. (1974) with minor modifications to optimize inhibition of the enzyme. The final incubation mixture (150 ll) contained 50 mM HEPES buffer (pH 7.5), 100 mM NaCl, 10 mM Mg

acetate, 50 lM dATP, 100 lM cAMP, 200 lM papaverine, 100 lM 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 lg membrane protein, and 1-2 X 10^6 cpm/assay tube of [a^{32}P]ATP (40 Ci/mmol). Reactions were initiated with the addition of [a^{32}P]ATP. Preliminary experiments showed that these concentrations of Mg^{++} (data not shown) and GTP (fig. 2.7) 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#C and 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, BioRad, Richmond, CA) and neutral alumina (Sigma), and samples were counted following 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.

(1951) following solubilization of the samples in 0.5 N NaOH. Crystalline bovine serum albumin was used as the standard.

D. Results

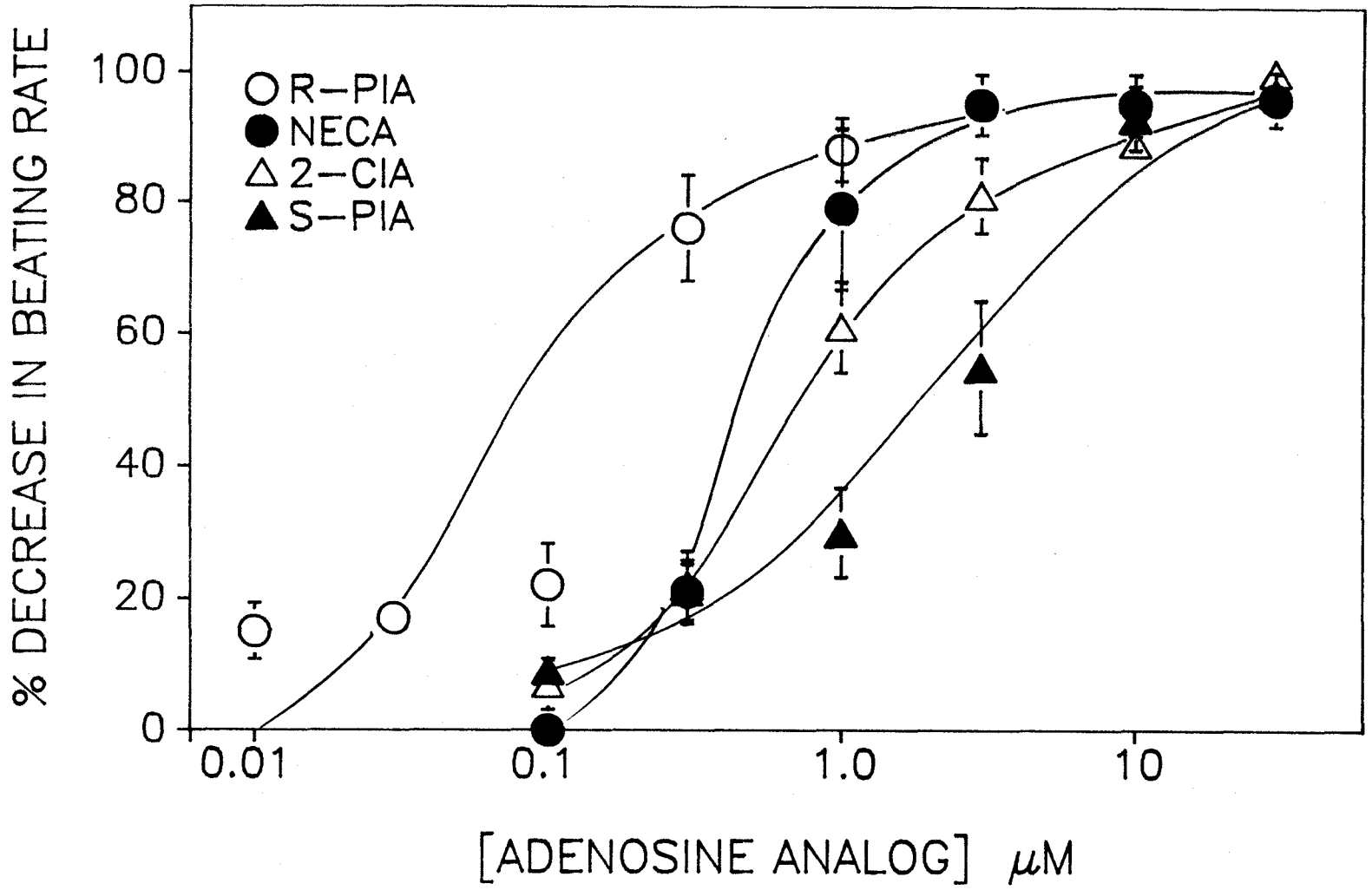
Pharmacological Response of Isolated Atria to Adenosine Analogs

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. 2.1. Control beating rates in the presence of the appropriate vehicle for the four groups in beats per minute were R-PIA 147 ± 8, NECA 153 ± 7, 2-ClA 129 ± 14 and S-PIA 149 ± 11 and 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 derived from analysis of the concentration-response data indicated that the rank order of potency was R-PIA (0.176 ± 0.065 1M) > NECA (0.516 ± 0.009 1M) >2-ClA (0.789 ± 0.079 1M) > S-PIA (2.94 ± 1.74 1M). 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 (Londos et al., 1980; Daly, 1983; Wolff et al. 1981).

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-pSPT on the response to 2-ClA was investigated. Because most methylxanthines are permeant to cell membranes and exert secondary effects that might

Figure 2.1 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 day-12 *in ovo* were determined. Each value represents the mean ± S.E. of 6 to 11 atria. The values are expressed as the mean percentage inhibition of the control beating rate.

Figure 2.1


% DECREASE IN BEATING RATE
100
80
60
40
20
0
R–PIA
NECA
2–CIA
S–PIA
0.01
0.1
1.0
10
[ADENOSINE ANALOG] μM

modify actions at surface receptors, 8-pSPT, a polar methylxanthine,was chosen for these beating rate experiments (Heller and Olsson, 1985). In the presence of 8-pSPT, 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-pSPT (Fig. 2.2). 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-pSPT of 0.29 1M.

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 1M. 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. 2.3). 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 1M during development, and did not exhibit any trend for an increased or decreased affinity as a function of ontogeny (Table 2.1). This indicates that although the maximal response obtained increased with age, the sensitivity of the atria to 2-ClA did not vary significantly.

Figure 2.2 Antagonism of the 2-ClA-induced inhibition of atrial beating rate by 8-p-sulfophenyltheophylline (8-pSPT). Spontaneous beating rates were determined in the presence of increasing concentrations of 2-ClA and vehicle ( ), 1 1M 8-pSPT ( ), 10 1M 8-pSPT ( ), or 100 1M 8-pSPT ( ). Each value represents the mean ± S.E. percentage inhibition of beating rate of 5-6 atria from embryos 16-days *in ovo*. Inset: Schild plot of 8-pSPT antagonism data.

Figure 2.2

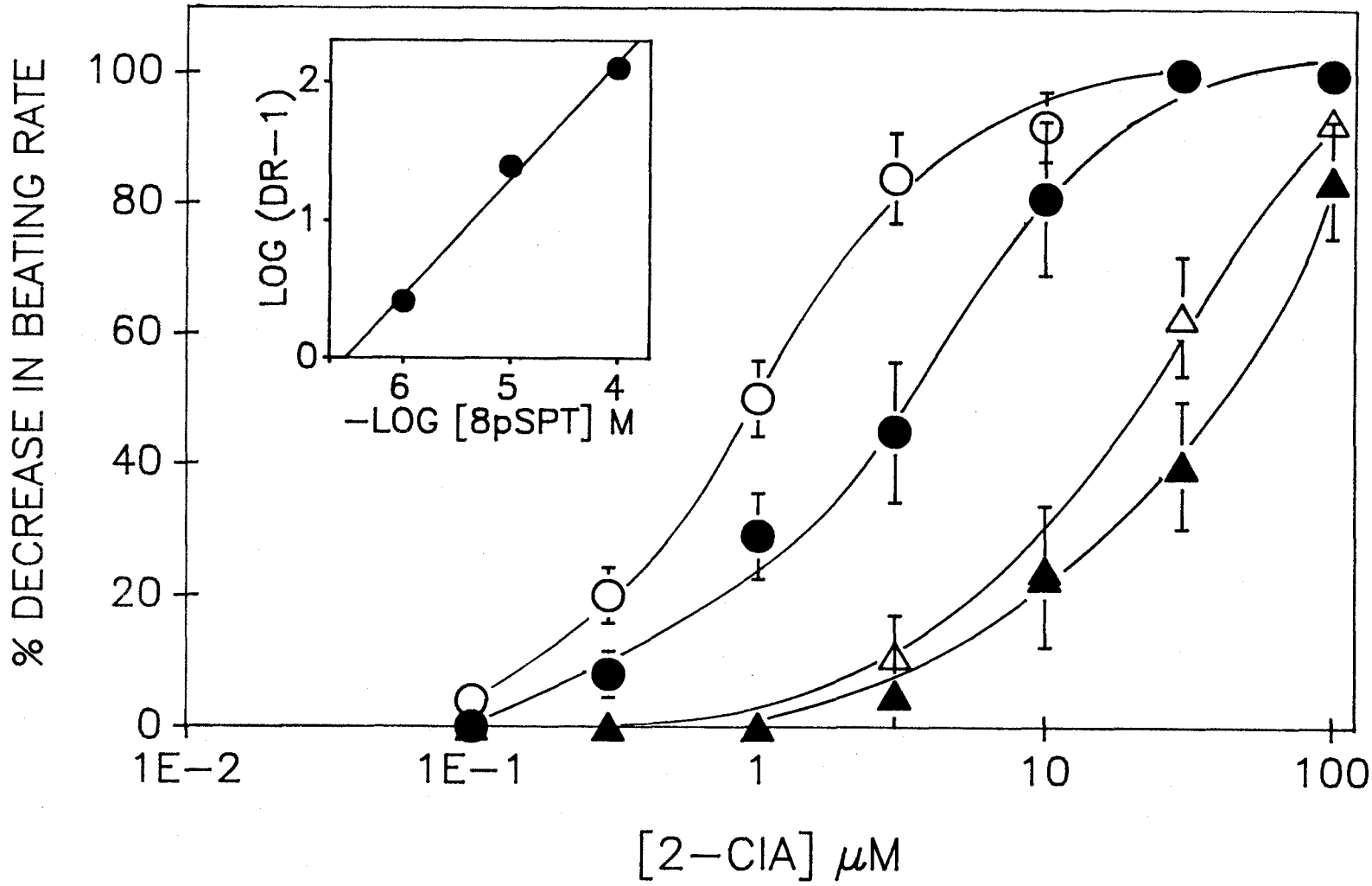

% DECREASE IN BEATING RATE
100
80
60
40
20
0
1E−2
1E−1
1
10
100
[2−ClA] μM
LOG (DR−1)
2
1
0
6
5
4
−LOG [8pSPT] M

Figure 2.3 Concentration - response curves for 2-ClA induced negative chronotropic effect as a function of embryonic age. Concentration - response data represent the mean ± S.E. 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.

Figure 2.3

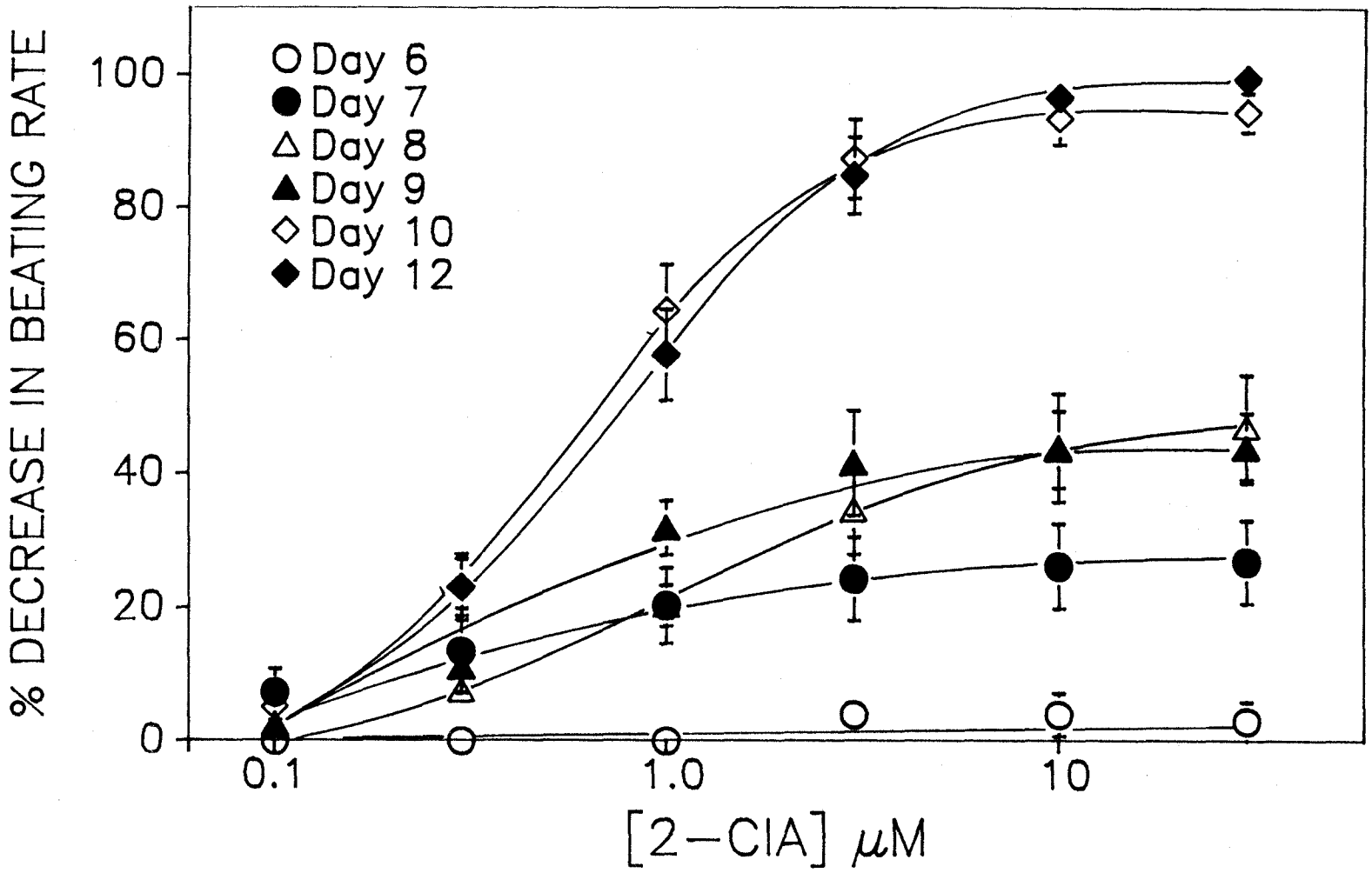

% DECREASE IN BEATING RATE
100
80
60
40
20
0
Day 6
Day 7
Day 8
Day 9
Day 10
Day 12
0.1
1.0
10
[2-ClA] μM

Table 2.1

Ontogeny of Responsiveness to 2-Chloroadenosine-Induced Negative Chronotropic Response in Embryonic Chick Atria[a]

Embryonic Age	EC_{50}	Maximum Inhibition of Atrial Beating Rate
days in ovo	1M	%
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)

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

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 Figure 2.4 [^{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 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 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 homogenous 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 Figure 2.5. The binding parameters derived from nonlinear regression analysis of [^{3}H]DPCPX saturation isotherms summarized in Table 2.2 demonstrate that the density of A_1 receptors increased approximately 2.5 fold between embryonic day 5 and 14. In contrast the Kd 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.

Agonist Binding to A_1 Adenosine Receptors

Figure 2.4 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 protein of [^{3}H]DPCPX specifically bound at a single, non-saturating 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±1.0; day-12, 26.8±1.6; day-14, 32.8±0.21; and day-16, 25.3±1.6.

Figure 2.4

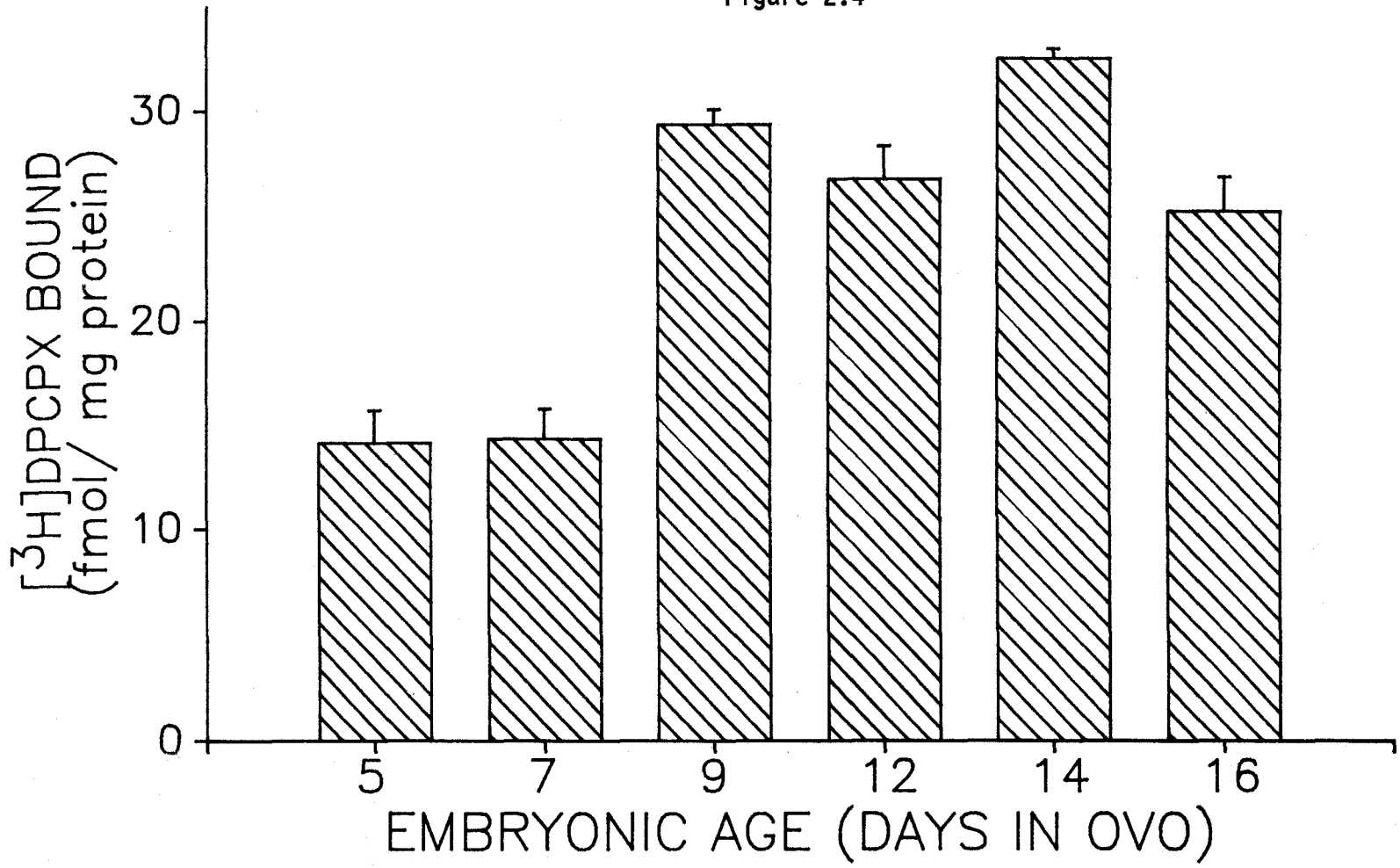

[3H]DPCPX BOUND
(fmol/ mg protein)
30
20
10
0
5
7
9
12
14
16
EMBRYONIC AGE (DAYS IN OVO)

Figure 2.5 A: Equilibrium saturation binding of [^{3}H]DPCPX to embryonic chick cardiac membranes. Membranes from embryonic day-7 and -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 Kd of 2.15 nM and a Bmax of 48.1 fmol/mg protein for embryonic day-7, and a Kd of 1.70 nM and a Bmax of 70.8 fmol/mg protein for embryonic day-14. Closed symbols indicate specific binding and open symbols indicate nonspecific binding. B: Scatchard replot of these saturation data.

Figure 2.5 A

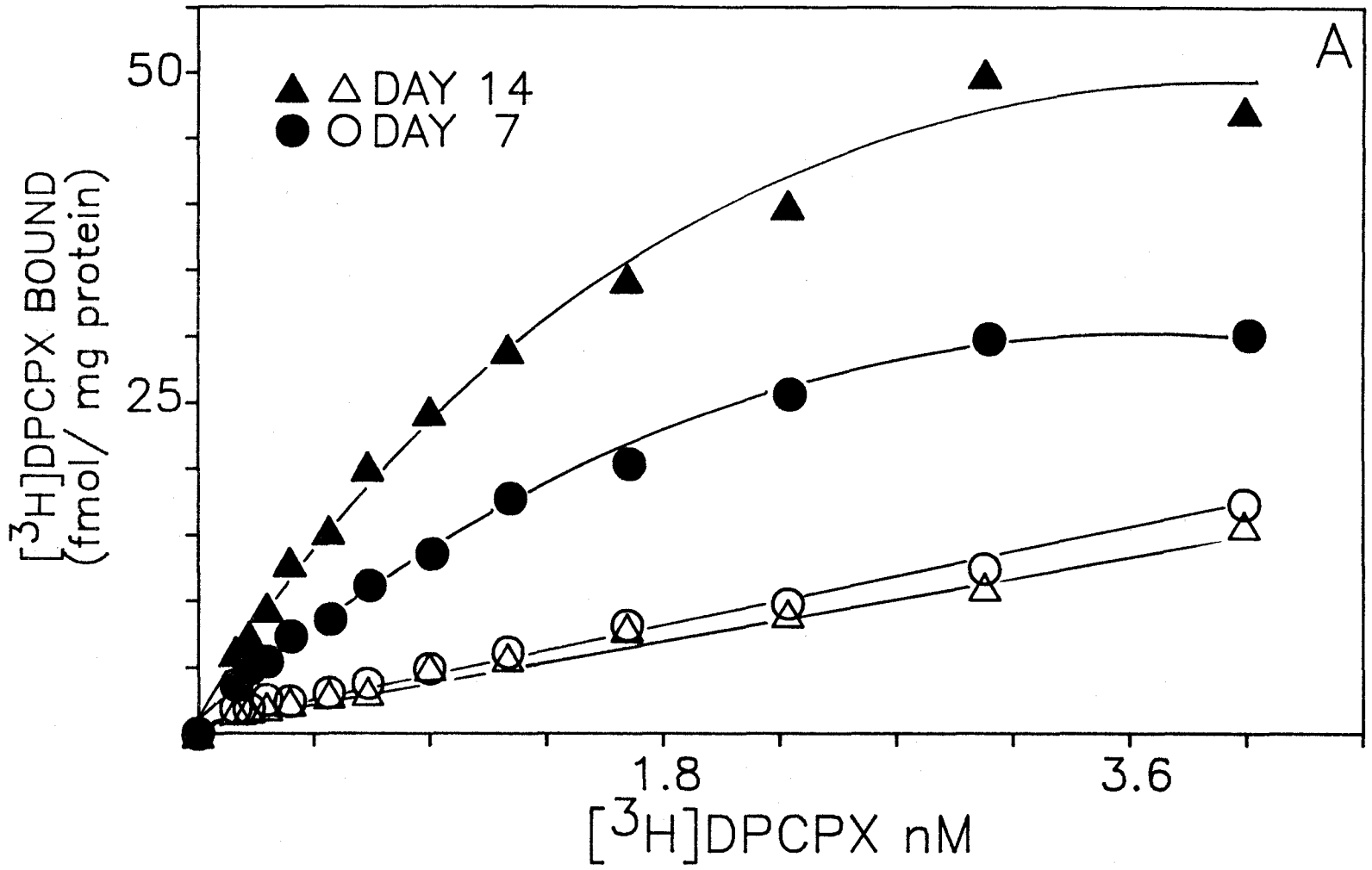

A
50
25
▲ △ DAY 14
● ○ DAY 7
[3H]DPCPX BOUND
(fmol/ mg protein)
1.8
3.6
[3H]DPCPX nM

Figure 2.5 B

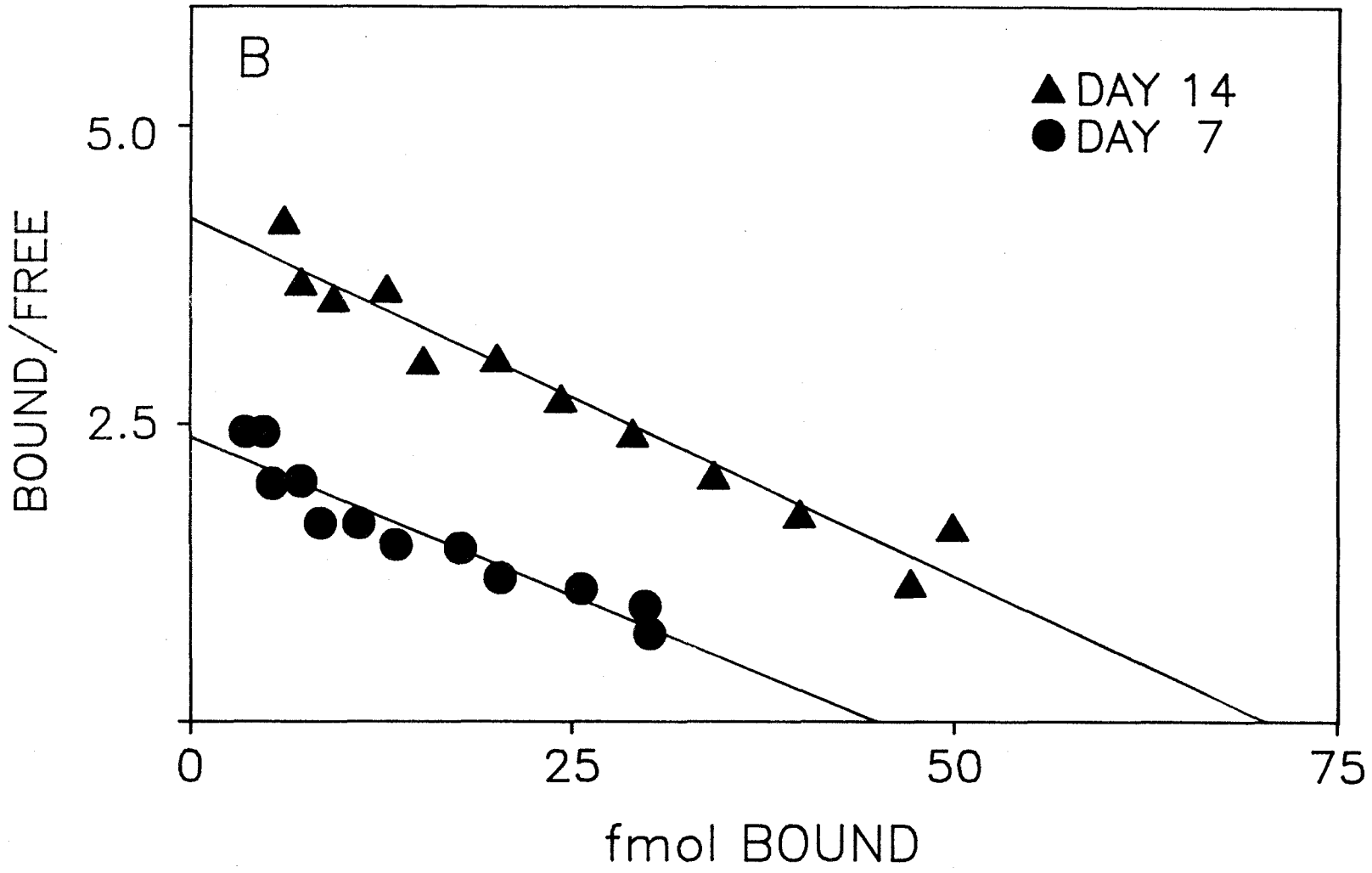


Table 2.2

Ontogeny of [^{3}H]DPCPX Binding to A_1 Adenosine Receptors in Embryonic Chick Hearts[a]

Embryonic Age	K_D	Bmax
days in ovo	nM	fmol/mg 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

[a]The values represent the mean ± S.E.M. 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 Bmax values were calculated as described under Materials and Methods.

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 GppNHp using [^{3}H]DPCPX and unlabeled cyclopentyladenosine (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 (Clark et al., 1987). Table 2.3 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 to 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 to 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

Table 2.3

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

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
	GppNHp	(3)	9.43 ---	26.5±26.5*	230.1±103	73.5±23.4*
Day 12	Control	(6)	1.79±0.3	46.8± 9.7	230.8±72.8	53.2±9.7
	GppNHp	(3)	7.27±1.0	38.5±19.7	158.9±53.8	61.5±19.7

Values presented are the mean ± S.E.M. for 3-7 individual experiments. K_H and K_L are, respectively, the high and low affinity equilibrium dissociation constants, while % R_H and % R_L represent the percentage of receptors in either the high or low affinity states. The inhibition 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 4 of 7 control experiments, whereas a two-site model significantly ($P < 0.01$) improved the fit in 6 of 6 control experiments in day 12 membranes. In contrast in the presence of 10 1M GppNHp in 2 of 3 experiments with embryonic day 6 membranes the data were adequately described by a one-site model of low affinity receptors.

*Statistically significant difference from the respective day 6 control values using t-Test for paired data ($P < 0.05$).

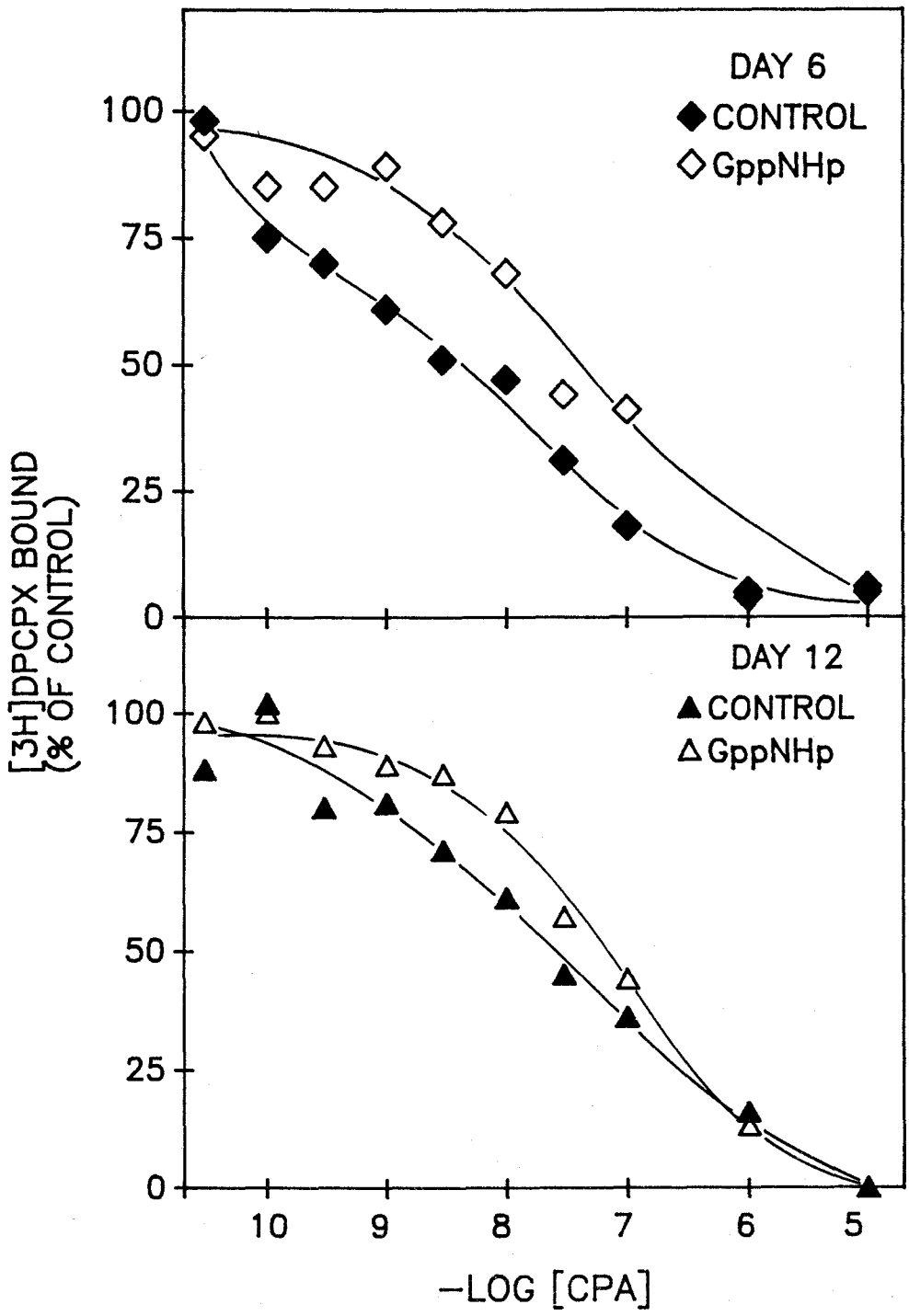
receptor-G protein coupling in embryonic day 12 membranes compared to that observed in embryonic day 6 hearts. To further characterize these differences in the coupling between A_1 adenosine receptors and G proteins at two developmental ages, guanine nucleotide regulation of CPA binding was examined in embryonic day 6 and 12 membranes. In the presence of 10 lM GppNHp the percentage of high-affinity sites was significantly ($p < 0.05$) reduced from 79.2% to 26.5% in embryonic day 6 membranes (Table 2.3). 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 GppNHp. In contrast, CPA binding to embryonic day 12 cardiac membranes was not significantly affected by the presence of 10 lM GppNHp (Table 2.3). This relative insensitivity of agonist binding to A_1 receptors of embryonic day 12 membranes is depicted in the representative competition experiments presented in Figure 2.6. 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 GppNHp-induced conversion to a low affinity state.

Adenosine Analog-Induced Inhibition of Adenylyl Cyclase Activity

Cyclopentyladenosine (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

Figure 2.6 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 1M GppNHp. 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 to embryonic day 6 membranes to the regulatory influence of 10 1M GppNHp.

Figure 2.6


DAY 6
CONTROL
GppNHp
DAY 12
CONTROL
GppNHp
[3H]DPCPX BOUND
(% OF CONTROL)
100
75
50
25
0
10
9
8
7
6
5
-LOG [CPA]

interactions with A_2 adenosine receptors present in the coronary vasculature which develops during this same embryonic period (Martinson et al., 1987, Manasek, 1979). The dependence of CPA-induced inhibition of adenylyl cyclase activity on GTP concentration is depicted in Figure 2.7. CPA did not affect adenylyl cyclase activity in the absence of GTP; however, at GTP concentrations greater than 0.1 1M the A_1 selective agonist inhibited activity. The EC_{50} for GTP-induced expression of this inhibitory modulation was 2.5 1M (Fig. 2.7 inset). In the presence of 100 1M 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. 2.8 DPCPX elicited a concentration dependent antagonism of the inhibition of adenylyl cyclase induced by 10 1M 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 significant differences in basal adenylyl cyclase activities observed between days 5 through 16 *in ovo* (Fig. 2.9). The maximum inhibition of basal adenylyl cyclase activity by CPA was approximately 10 to 15% and was invariant during this period of embryonic development (Figure 2.9). 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

Figure 2.7 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 1M CPA. Data points represent the mean values ± S.E. of triplicate determinations from a single experiment. The experiment was replicated twice with similar results. Inset: The percentage inhibition of basal adenylyl cyclase activity produced by 100 1M CPA is plotted as a function of GTP concentration. The maximal percentage inhibition by CPA was 17% in this experiment and occurred at a GTP concentration of 100 1M.

Figure 2.7

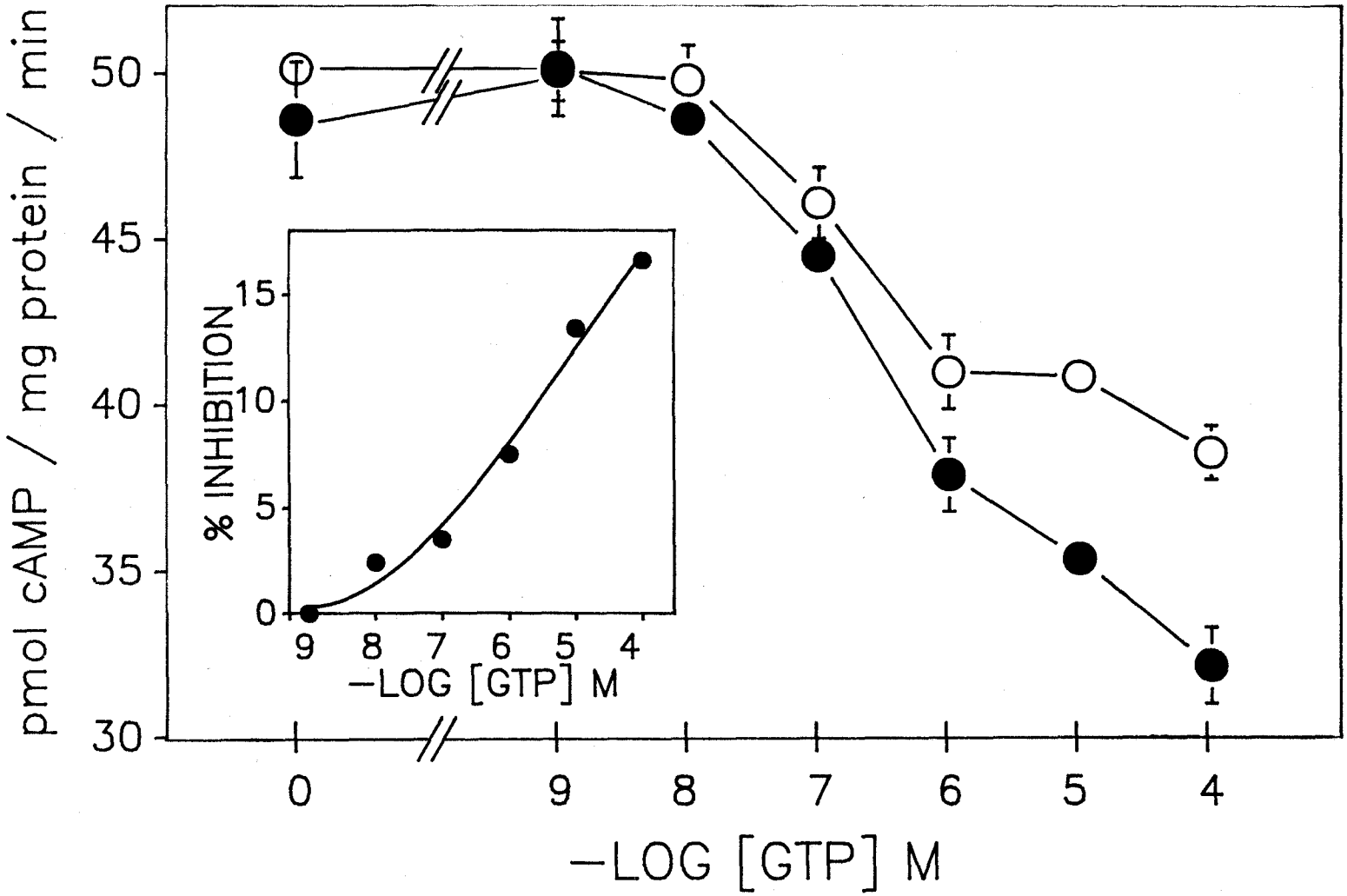

pmol cAMP / mg protein / min
50
45
40
35
30
0
9
8
7
6
5
4
−LOG [GTP] M
% INHIBITION
15
10
5
0
9
8
7
6
5
4
−LOG [GTP] M

Figure 2.8 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 1M CPA. Data points represent the mean values ± S.E. of triplicate determinations from a single experiment. The experiment was replicated twice with similar results.

Figure 2.8

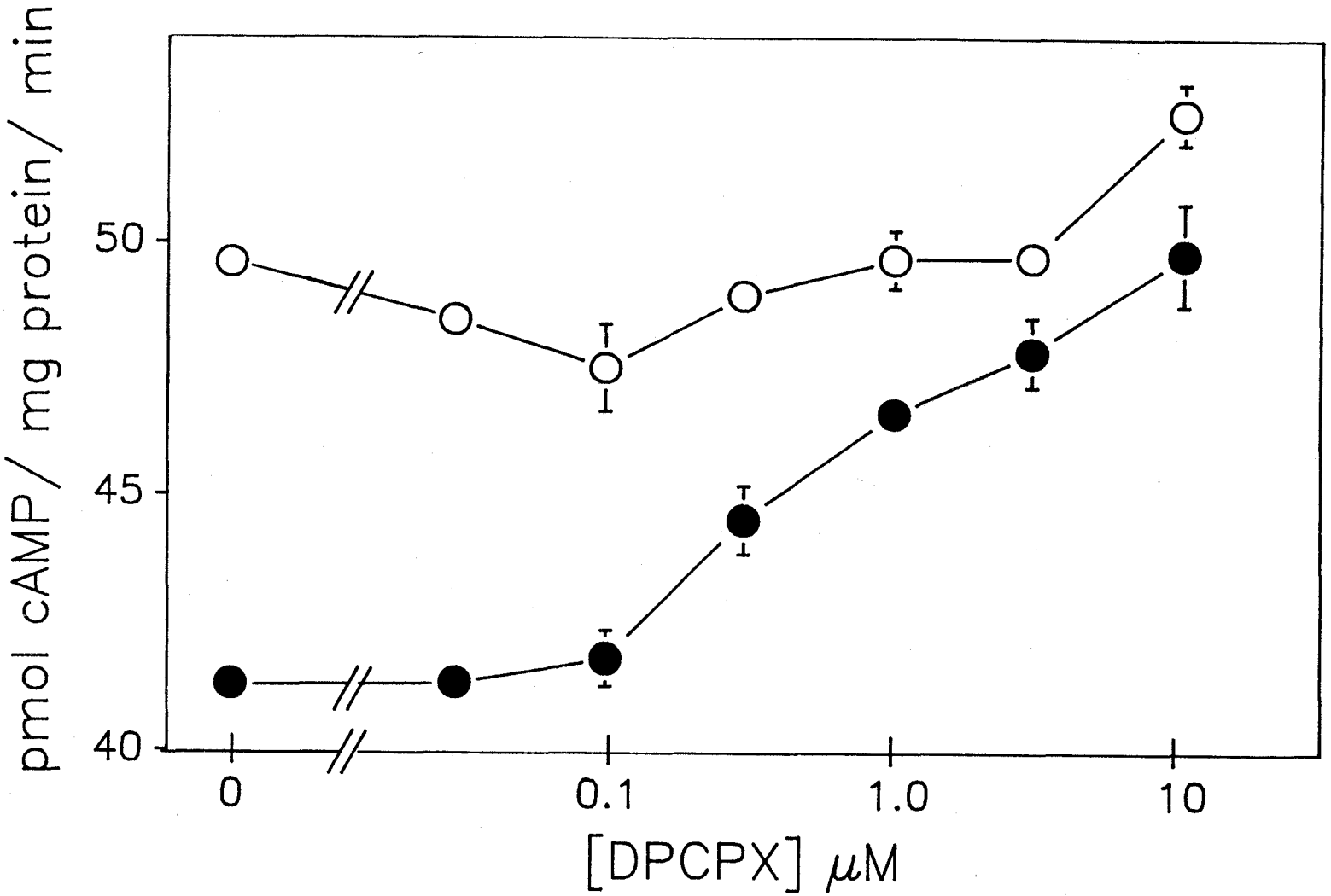
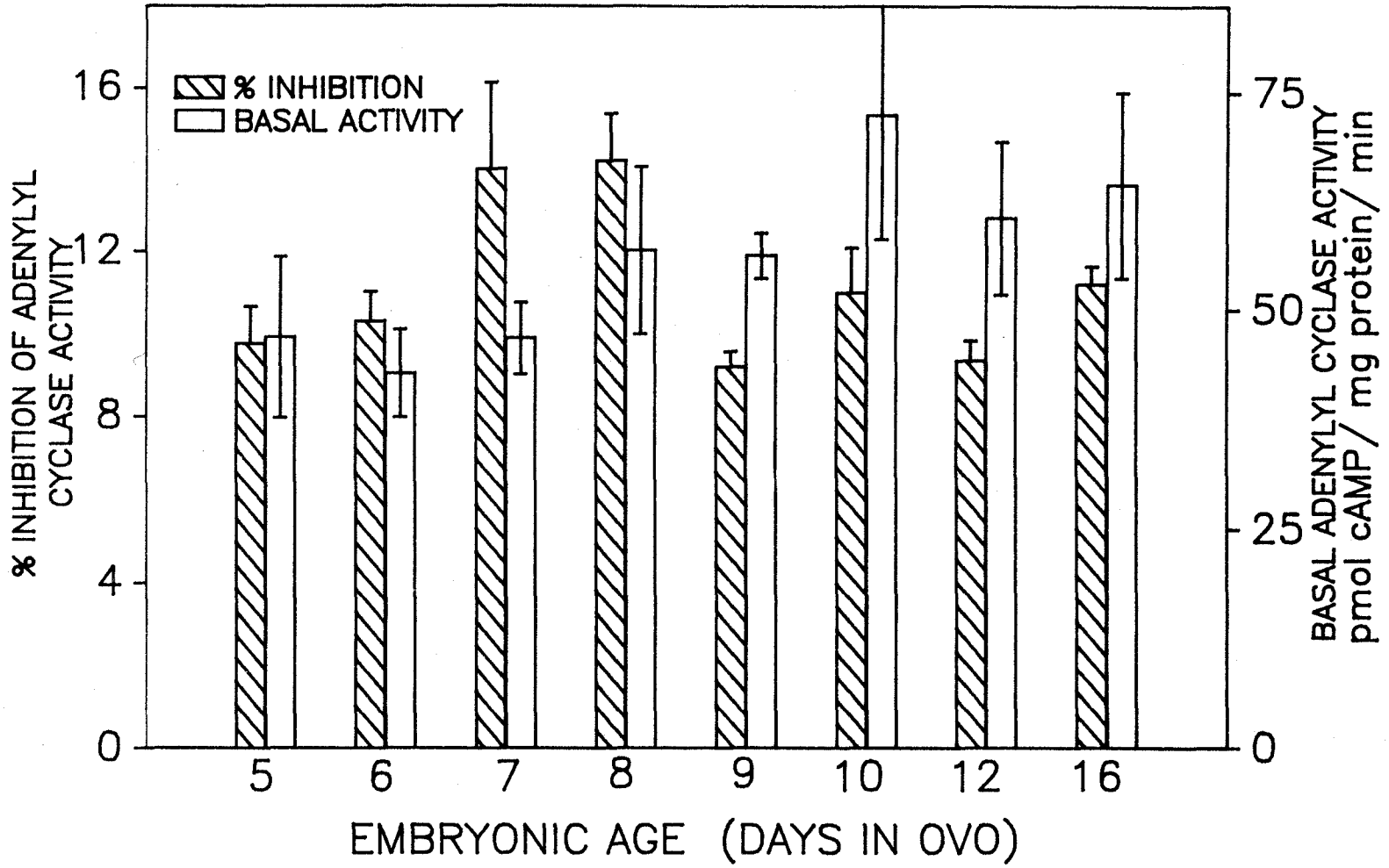


Figure 2.9 Developmental profile of basal adenylyl cylcase activity and maximal percentage of inhibition of adenylyl cyclase by CPA in embryonic chick heart membranes. Cardiac membranes prepared from embryos of the ages indicated were assayed for adenylyl cyclase activity in the presence of 100 1M GTP. Bar heights represent either the percentage inhibition of basal activity in response to 10 1M CPA or basal adenylyl cyclase activity in pmol cAMP/mg protein/min. Each value is the mean ± S.E. of 2-3 separate determinations for each embryonic age.

Figure 2.9


% INHIBITION
BASAL ACTIVITY
% INHIBITION OF ADENYLYL CYCLASE ACTIVITY
BASAL ADENYLYL CYCLASE ACTIVITY pmol cAMP/ mg protein/ min
0
4
8
12
16
0
25
50
75
5
6
7
8
9
10
12
16
EMBRYONIC AGE (DAYS IN OVO)

from unresponsive to fully responsive. Representative concentration curves for CPA-induced inhibition of adenylyl cyclase activity in 6, 8-, 10- and 16-day embryonic chick heart membranes are depicted in Figure 2.10. The IC_{50} values (± S.E.M.; n=3 for each embryonic age) for inhibition of adenylyl cyclase by CPA ranged from 0.91 ± 0.28 1M at day 6 to 0.39 ± 0.16 and 0.59 ± 0.10 1M at day 10 and 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 (Figure 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.

Figure 2.10 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.

Figure 2.10

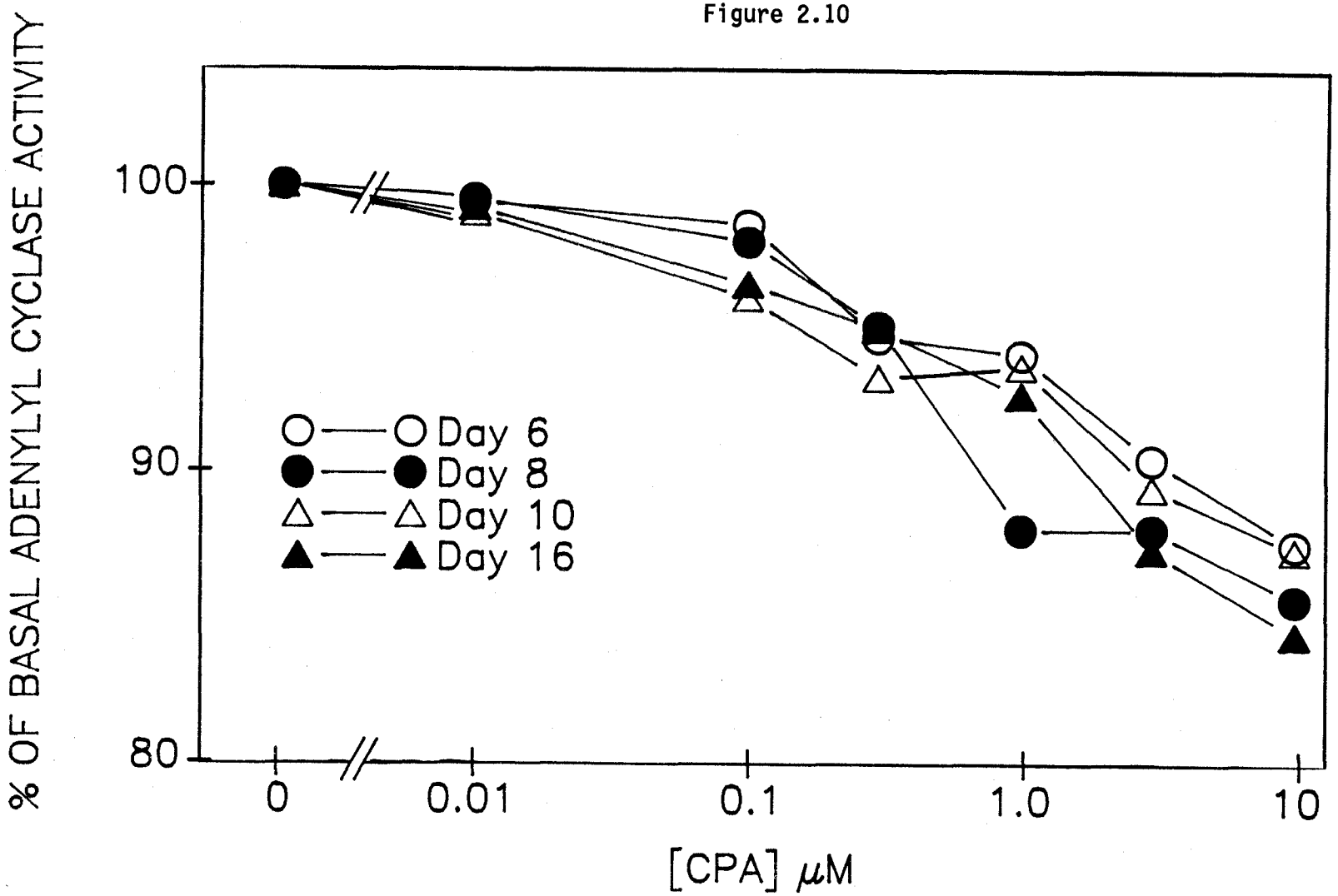
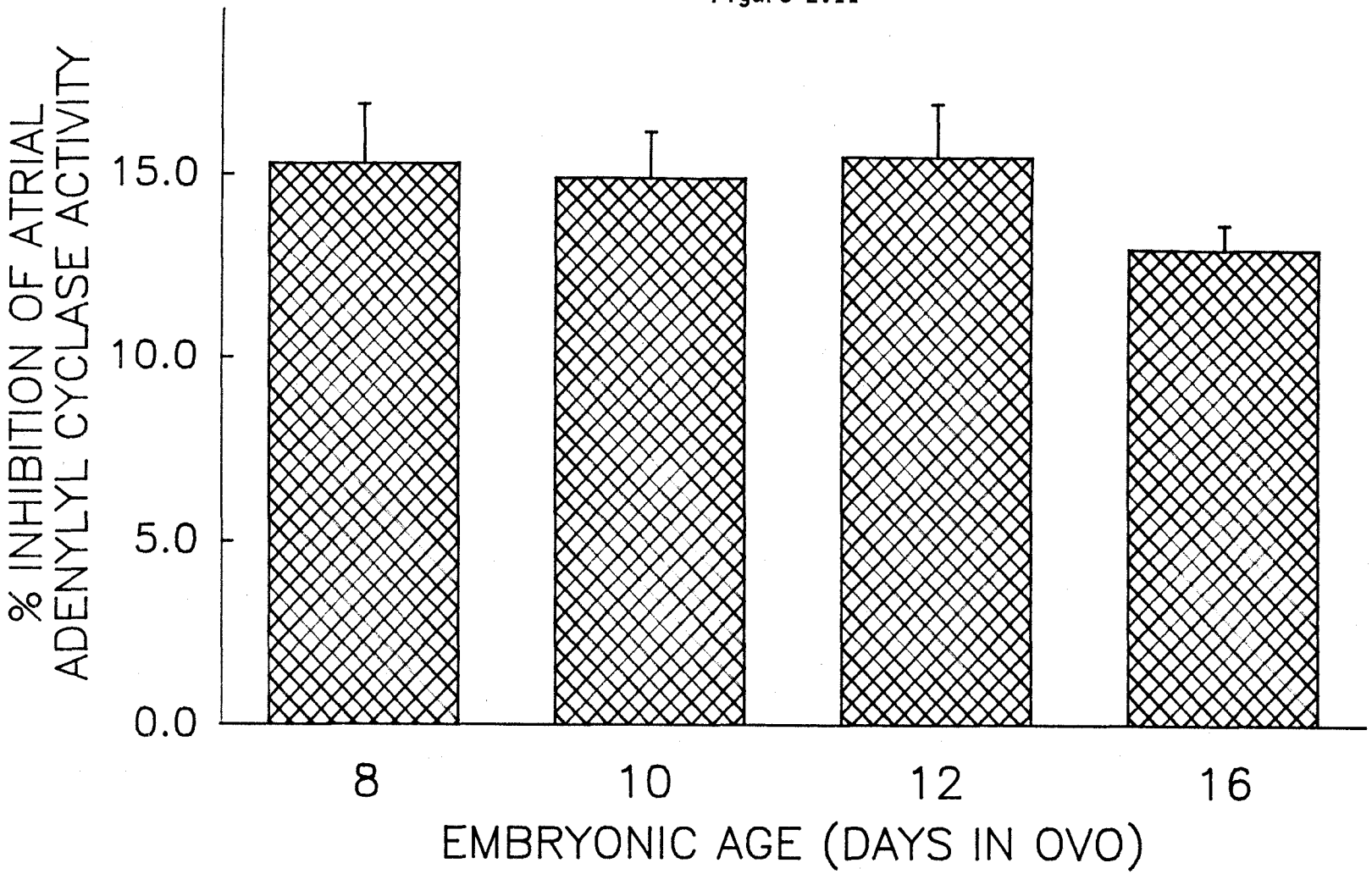

% OF BASAL ADENYLYL CYCLASE ACTIVITY
100
90
80
0
0.01
0.1
1.0
10
[CPA] μM
Day 6
Day 8
Day 10
Day 16

Figure 2.11 Developmental profile of maximal percentage of 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 1M GTP. Bar heights represent the percentage inhibition of basal activity in response to 10 1M CPA. Each value is the mean ± S.E. of 3 separate determinations for each embryonic age depicted.

Figure 2.11


% INHIBITION OF ATRIAL ADENYLYL CYCLASE ACTIVITY
15.0
10.0
5.0
0.0
8
10
12
16
EMBRYONIC AGE (DAYS IN OVO)

E. 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 (Hopwood, et al., 1987). 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 pharmacologic sensitivity occurred on embryonic day 7 as evidenced by a 27% reduction in atrial beating rate in the presence of 2-ClA (30 1M). 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 8-p-sulfophenyltheophylline 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.

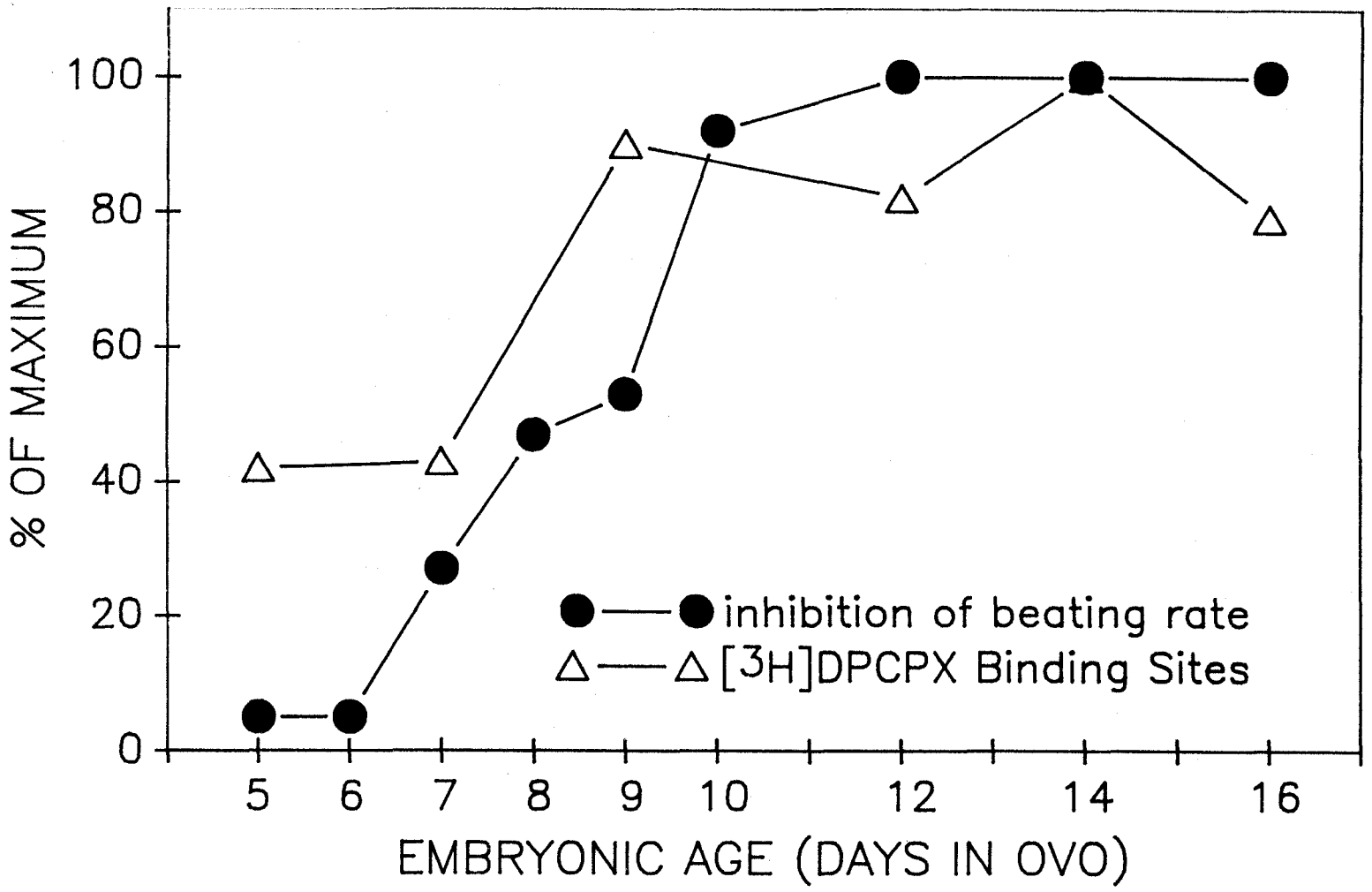
In order to evaluate whether the developmental increase in pharmacological sensitivity to 2-ClA reflected changes in the number of A_1 adenosine receptors, the ontogenesis of A_1 adenosine receptors was assessed using the antagonist radioligand [^{3}H]DPCPX as a probe (Leid et al., 1988). DPCPX is an adenosine receptor antagonist with a high

selectivity for A_1 over A_2 receptor subtypes as demonstrated by its ability to selectively block the negative chronotropic action and not the coronary vasodilator action of R-PIA in isolated rat heart (Hosey et al., 1984). The relationship between the developmental profiles for pharmacological sensitivity of the chick atrium to 2-ClA and A_1 adenosine receptor number is depicted in Figure 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 pharmacologic 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 of the atria and ventricle.

In studies of the development of chick heart muscarinic receptors Halvorsen and Nathanson (1984) 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

Figure 2.12 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 protein), while values for sensitivity to the negative chronotropic response to 2-ClA are from the data in Table 1.

Figure 2.12


100
80
60
40
20
0
% OF MAXIMUM
5
6
7
8
9
10
12
14
16
EMBRYONIC AGE (DAYS IN OVO)
inhibition of beating rate
[3H]DPCPX Binding Sites

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 to 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 suggests 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 (Green, 1987; Parsons and Stiles, 1987).

The appearance of pharmacologically inactive A_1 adenosine receptors in day-5 and -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 cylcase 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 days 5 through 16. In accordance with the results of Halvorsen and Nathanson (1984), 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 guanine nucleotide binding proteins occurs early in embryonic development. However, unlike muscarinic receptor mediated negative chronotropy which is nearly fully responsive in embryonic day 5 atria, the physiologic sensitivity to A_1 adenosine receptor activation is not maximally expressed until embryonic day 12.

In agreement with the findings of Linden et al. (1985) 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 cylcase has been attributed to the greater density of muscarinic than A_1 adenosine receptors in rat heart (Linden et al., 1985). 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 (Liang et al., 1986; Hosey et al., 1985). 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 (Leung et al., 1985), 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 physiologic 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 guanine nucleotide regulatory protein in guinea pig atrial tissue (Kurachi et al., 1986). Moreover, adenosine and muscarinic receptors have been shown to share the same pool of cardiac K^+ channels in a single cell (Kurachi et al., 1986). 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 guanine nucleotide regulatory proteins.

Although a convergence of adenosine receptors and muscarinic receptors on a single pool of K^+ channels has been shown, the ontogenetic profiles for physiologic sensitivity to the negative chronotropic effects of muscarinic and adenosine agonists in embryonic chick hearts differs. Whereas isolated chick atria are nearly fully responsive to carbachol-induced inhibition of beating rate by day-5 *in ovo* (Halvorsen and Nathanson, 1984), 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 6 may indicate that the functional coupling of these recognition sites to K^+ channels via guanine nucleotide binding proteins is inoperative during this developmental period. Additional investigation of the developing chick heart will be required to more fully define the molecular mechanisms which underlie the A_1 adenosine receptor mediated negative chronotropic response.

Acknowledgments

The authors gratefully acknowledge the excellent word processing assistance of Ms. Elaine Luttrull. This research was supported in part by a grant from the Oregon Affliate of the American Heart Association. Permission to include this paper in the thesis was given by Williams and Wilkins Publishers and Molecular Pharmacology (Molecular Pharmacology, 35:661-670, 1989).

ABBREVIATIONS USED

2-ClA, 2-chloroadenosine; CPA, N^6-cyclopentyladenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; [^{3}H]DPCPX, 8-cyclopentyl-1-1,3[^{3}H]dipropylxanthine; G_i, GTP-binding protein which mediates inhibition of adenylyl cyclase; G_o, GTP-binding protein distinct from G_i; Gpp(NH)p, guanyl-5'-yl-imidodiphosphate; NECA, 5'-N-(ethylcarboxamido)adenosine; R-PIA, N^6-(R-phenylisopropyl)adenosine; S-PIA, N^6-(S-phenylisopropyl)- adenosine; 8pSPT, 8-(p-sulfophenyl)theophylline.

CHAPTER 3

Cyclopentyladenosine-Induced Down Regulation of A_1 Adenosine Receptors in Embryonic Chick Heart

T. Ann Blair and Thomas F. Murray

A. Summary

Previous investigations utilizing embryonic chick atria have indicated that adenosine analogs elicit a negative chronotropic effect via an A_1 adenosine receptor. In the present study we have employed the developing chick heart as a model system to investigate the regulation of A_1 adenosine receptors. Sustained activation of adenosine receptors was accomplished with *in ovo* injections of the A_1-selective adenosine agonist cyclopentyladenosine (CPA). Treatment with CPA resulted in a decreased A_1 adenosine receptor number as measured by the specific binding of the A_1-selective antagonist radioligand 8-cyclopentyl-1,3-[^{3}H]dipropylxanthine ([^{3}H]DPCPX). The binding parameters derived from nonlinear regression analysis of [^{3}H]DPCPX saturation isotherms indicated that *in ovo* treatment with 1 lmol CPA produced a 46 $\pm$ 11% reduction in the density of A_1 receptors in cardiac membranes. The K_d value for [^{3}H]DPCPX binding to adenosine receptors in CPA-treated membranes was not significantly different from the saline-treated value (CPA 2.9 $\pm$ 0.1 nM; saline 3.1 $\pm$ 0.9 nM). The decrease in receptor number was dose- and time-dependent. The maximum dose of CPA (10 lmol) resulted in a 77% decrease in the density of adenosine receptors with an ED_{50} for the CPA-induced down regulation of 0.5 lmol. Administration

in ovo of 1 lmol CPA resulted in a decrease in the number of [^{3}H]DPCPX binding sites which was significant at 4 hours, reached a maximum value at 8 hours, and was maintained throughout the time span evaluated (24 hr). Co-injection of theophylline was able to attenuate the decrease in adenosine number induced by 1 lmol CPA. Down regulation of the cardiac A_1 adenosine receptor was accompanied by a decrease in the ability of CPA to elicit a receptor-mediated negative chronotropic response. The ability of CPA to reduce cAMP content in whole heart following down regulation of the A_1 adenosine receptor was not significantly changed. Agonist/antagonist competition data were consistent with the presence of two populations of adenosine receptors with differing affinities for agonists. Down regulation of A_1 receptor density occurred in the absence of any effect on the distribution of receptors between the high and low agonist affinity states. These data suggest chronic exposure of embryonic day 9 chick hearts to an adenosine analog effects a down regulation of A_1 adenosine receptors that is associated with a diminished physiological response to receptor stimulation.

B. Introduction

The cardiac actions of adenosine are initiated by the binding of adenosine or adenosine receptor agonists to A_1 receptors located on the myocardial membrane (Collis, 1983, Blair, 1989). The functional role of adenosine in this tissue appears to be primarily a modulatory one as evidenced by negative chronotropic (Drury et al., 1929, Hatae et al., 1989) dromotropic (Belardinelli et al. 1983) and inotropic (Dobson

1983) effects. A_1 receptors have been shown to be coupled to the inhibition of adenylyl cyclase in cardiac tissue (Schutz et al., 1986, Leung et al., 1986) in addition to coupling in a stimulatory manner to a specific class of potassium channels in atrial tissue (Jochem et al., 1983, Kurachi et al., 1986). Both of these actions occur via pertussis toxin sensitive guanine nucleotide-binding proteins and are believed to be the underlying mechanisms by which adenosine elicits its cardiac depressant actions (Bohm et al., 1989, Endoh et al., 1983).

Prolonged or repeated exposure of tissues and cells to a variety of hormones or drugs often leads to a decrease in the number of receptors accompanied by a decrease in biochemical and/or physiological responsiveness to subsequent exposure (Clark, 1986). This phenomenon has been well characterized in receptor systems that activate adenylyl cyclase such as the b-adrenergic receptor system (Sibley and Lefkowitz, 1985; Harden et al., 1983). Much less is known about the mechanisms by which receptor systems that couple to the inhibition of adenylyl cyclase down regulate receptors in response to agonist exposure. Desensitization of the A_1 adenosine receptor-adenylyl cyclase system in rat adipocytes has recently been described (Hoffman et al., 1986; Parsons and Stiles, 1987; Green, 1987). These studies demonstrated an agonist-induced reduction in agonist radioligand binding concomitant with decreased responsiveness in associated effector systems. Most recently, Shryock et al. (1989) have demonstrated a decrease in agonist radioligand binding to A_1 receptors following *in ovo* agonist exposure in embryonic chick heart which was correlated with desensitization of the negative inotropic response. To further characterize the effects

of chronic *in ovo* exposure of the embryonic chick heart to adenosine receptor agonists, we have utilized an antagonist radioligand to evaluate the down regulation of A_1 adenosine receptor binding sites at several stages of development. We have also characterized the biochemical and physiological consequences of *in ovo* exposure to an adenosine receptor agonist by examining possible alterations in the responsiveness of A_1 receptor mediated regulation of cAMP levels and the negative chronotropic response associated with A_1 receptor activation. To evaluate the role of A_1 receptor-G protein interactions in this process we utilized cyclopentyladenosine as an agonist in competition experiments with [^{3}H]DPCPX.

C. Experimental Procedures

Materials

White Leghorn chick embryos were obtained locally from the Poultry Science Department, Oregon State University (Corvallis, OR) and were maintained at 39^0 in a humidified incubator. The embryonic ages were determined by comparison with the description by Hamburger and Hamilton (1951). Chemicals were obtained from the following sources: GTP was purchased from Sigma Chemical Co. (St. Louis, MO); Adenosine deaminase from Boehringer-Mannheim (Mannheim, West Germany); [^{3}H]DPCPX from Amersham (Chicago, IL); and CPA from Research Biochemicals Incorporated (Wayland, MA).

In ovo injections

The injection sites were swabbed with 70% ethanol and injections

made using a 1 cc tuberculin syringe and 25 g needle. One hole was placed over the embryo and the second placed over the air sac to allow space for the injection volume. Following the injections the holes were sealed with tape and the eggs returned to the incubator.

Each treatment group received doses of the appropriate drug on day 9 *in ovo* (unless specified otherwise) and subsequent analyses were performed 24 $\pm$ 2 hr later for all experiments except those in which the time course was being determined. Drugs were dissolved in phosphate buffered saline (PBS, 40.4 mM Na_2HPO_4, 9.5 mM KH_2PO_4, 200 mM NaCl, pH 7.4) and were injected in a volume of 100 ul. CPA concentrations greater than 3 lmol required the addition of 0.1% ethanol to the vehicle. Control embryos received 100 ul PBS or PBS + 0.1% ethanol injections. The use of this technique resulted in a mortality rate for drug treated embryos which was not significantly different from that of PBS injected controls. In embryos receiving doses of CPA > 3 lmoles or doses of theophylline > 10 mmoles the mortality rate was slightly increased (~10%).

Atrial beating rate studies

Measurement of atrial beating rate was performed as previously described with minor modifications (Blair et al., 1989). Intact beating hearts were removed from saline- and CPA-treated chick embryos and placed in beating rate medium (149 mM NaCl, 5.9 mM KCl, 1.8 mM CaCl2, 10 mM Mg acetate, 10 mM HEPES, 2.3 mM glucose, and 0.4 mM Na2HPO4, adjusted to pH 7.4) that was maintained at 37^{o} and aerated with 100% O2. Atria were separated from the ventricles, pinned to a

Sylgard-coated Petri dish containing 10 ml of beating rate medium and allowed to equilibrate for one hour. During this equilibration period the beating rate medium was replaced with fresh medium every 15 min to insure the removal of CPA bound during *in ovo* exposure. Four to six atria from both groups were prepared and those demonstrating a stable rhythm with a beating rate between 18 and 32 beats/10 sec were used for each experiment. Beating rates were determined visually with a dissecting microscope by counting beats during three or four 10-sec epochs, 60 sec apart. CPA, a selective A_1 agonist, was used to determine the negative chronotropic response to A_1 receptor stimulation. There was no evidence for the development of desensitization during the time interval evaluated (up to eight min). Following each dose response experiment the atria were washed three times with fresh medium and allowed to equilibrate for 30 min. Beating rates were then determined and compared with the initial baseline beating rates to establish reversibility of the drug response. Cumulative concentration-response experiments were performed on 8 to 12 atria at embryonic ages day 8, 10 and 12 *in ovo*.

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

Tissue preparation

Intact beating hearts were removed from chick embryos, great vessels removed, and placed in ice-cold 50 mM Tris buffer (pH 7.5 at 4^{o}). The hearts were homogenized in 20 volumes of 10 mM Tris and 10 mM

EDTA with 10-12 strokes in a Dounce homogenizer (B pestle). The homogenate was filtered through two layers of gauze and centrifuged at 35,000 x g for 10 min at 4^{o}. The supernatant was discarded and the pellet resuspended in 30 volumes of 50 mM Tris and 1 mM EDTA using a Dounce homogenizer (A pestle). This suspension was recentrifuged as above and the resultant pellet resuspended in a buffer containing 1 mM EDTA, 100 mM NaCl, 100 uM GTP, and 7.5 IU/ml adenosine deaminase and incubated at 37^{o} for 30 min to promote dissociation and metabolism of membrane-derived adenosine. This preincubation was followed by three centrifugation (35,000 x g for 10 min) and resuspension steps to insure complete removal of CPA bound during *in ovo* exposure. To verify the removal of CPA administered *in ovo*, [^{3}H]cyclohexyladenosine ([^{3}H]CHA), a selective A_1 agonist congener of CPA, was injected *in ovo* and the embryo incubated for 24 hr. [^{3}H]CHA is similar to CPA in its binding characteristics and physiochemical properties and thus should mimic the distribution of CPA injected *in ovo* (Williams et al., 1986). The amount of radioactivity present at each step of the membrane preparation was monitored and found to be below detectable levels subsequent to the first wash indicating that the membrane preparation afforded virtually a complete removal of CPA (data not shown). The final pellet was resuspended in 5 to 20 volumes (depending on embryonic age) of ice-cold 50 mM Tris buffer, to obtain a protein concentration of 1 to 2 mg/ml, and kept on ice until used in the radioligand binding assay.

A_1 adenosine receptor binding assay

The specific binding of the A_1-selective antagonist ligand

[^{3}H]DPCPX (120 Ci/mmol) to cardiac membranes was determined as previously described (Blair et al., 1989). Briefly, aliquots (175 ul) of the cardiac membrane preparation were incubated for 90 min at 22^o with 25 ul of [^{3}H]DPCPX, 25 ul of 100 lM GTP or Tris buffer, and 25 ul of competing compound or Tris buffer, in a total volume of 250 ul. The binding reactions were terminated by the addition of 2 ml of ice-cold Tris buffer, followed by filtration under vacuum of the assay tube contents over Schleicher and Schuell #32 filter strips, which had been presoaked in 0.5% polyethyleneimine (Sigma), using a Brandel cell harvester (Model M-24R; Brandel Instruments, Gaithersburg, MD). Filters were then rinsed with four x 4-ml washes of ice-cold Tris buffer to remove unbound radioactivity. Filter disks were place into counting vials to which 3.5 ml of Biocount scintillation cocktail (RPI Corp. Mont 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 or 100 uM CPA and represented approximately 70% of the total binding at the K_d value for [^{3}H]DPCPX. Saturation isotherm data were analyzed using the iterative curve-fitting program FITSAT on the PROPHET system. In all equilibrium saturation binding experiments, the [^{3}H]DPCPX isotherms were adequately described by a one-site model. Competition experiments were analyzed using the iterative public procedure FITCOMP on the PROPHET computer system.

Cyclic AMP assay

Intact beating hearts were removed from saline- and CPA-treated chick embryos and placed in beating rate medium that was maintained at 37^{0} and aerated with 100% O_2. The whole hearts were allowed to equilibrate for one hour during which time the beating rate medium was replaced every 15 mins to insure removal of CPA bound during *in ovo* exposure. Subsequent to the equilibration period, the hearts were exposed to PBS or 10 1M CPA. After 15 mins the hearts were quickly removed, blotted, frozen and stored at -70^{0} until assayed for cAMP content. Cyclic AMP was extracted from the tissue using a modification of the method of Covents et al. (1989). The frozen tissue was weighed and placed in 8 volumes of ice cold 4 mM EDTA, pH 7.4. The sample was sonicated for 20 secs with a Tekmar Sonic Disruptor and then boiled for six min. Denatured proteins were removed by centrifugation at 15,800 x g for 10 mins. Cyclic AMP concentrations were quantitated with a radioimmunoassay using ^{125}I-cAMP as the labelled tracer.

Protein determination

Membrane protein content was assayed by the method of Lowry et at. (1951) after solubilization of the samples in 0.5 N NaOH or the method of Smith et al. (1985). Crystalline bovine serum albumin was used as the standard.

D. Results

Effects of CPA exposure *in ovo* on binding of [^{3}H]DPCPX to adenosine receptors

The *in ovo* exposure to 1 lmole of the selective A_1 adenosine agonist CPA to chick embryos led to a decrease in the binding of [^{3}H]DPCPX to cardiac membranes. The observed agonist-induced reduction in the specific binding of [^{3}H]DPCPX could be the result of either a change in the affinity of the adenosine receptor for [^{3}H]DPCPX or a decrease in the total receptor number. To differentiate between these two possibilities, equilibrium saturation analysis was performed in cardiac membrane preparations derived from embryos pretreated *in ovo* with CPA or saline on embryonic day 9. Following a 24 hour *in ovo* incubation period cardiac membranes were prepared and the specific binding of [^{3}H]DPCPX was found to be of high affinity, saturable, and to a homogeneous population of recognition sites in both groups. Representative saturation isotherms and corresponding Scatchard replots are shown in Fig. 3.1, A and B. The binding parameters derived from nonlinear regression analysis of [^{3}H]DPCPX saturation isotherms revealed a 46 $\pm$ 11% decrease in the density of A_1 receptors in the CPA treatment group as compared to the saline injected control group. In contrast no significant changes in the apparent affinity of the A_1 adenosine receptor for [^{3}H]DPCPX were observed (K_d 2.9 $\pm$ 0.1 nM for CPA treated; 3.1 $\pm$ 0.9 nM for control). Similar results were obtained from experiments with cardiac membranes derived from embryonic day 5 and 14 hearts (data not shown).

Time-response and dose-response relationships of CPA-induced decrease in [^{3}H]DPCPX binding sites

The injection of 1 lmol CPA *in ovo* to day 9 chick embryos led to

Figure 3.1. A, Equilibrium saturation binding of [^{3}H]DPCPX to embryonic chick cardiac membranes. Membranes from embryonic age day 10 treated *in ovo* with saline or 1 lmol CPA at embryonic age day 9 were incubated with the indicated concentrations of ^{3}H]DPCPX. Values shown are from a single representative experiment, which was replicated 2 times. The fit shown was obtained using Lundon I saturation analysis software, which yielded a K_d of 2.3 $\pm$ 0.3 nM and a B_{max} of 60.1 $\pm$ 3.7 fmol/ mg protein for saline treated embryos and a K_d of 2.8 $\pm$ 0.7 nM and a Bmax of 39.0 $\pm$ 5.7 fmol/ mg protein for CPA treated embryos. *Closed symbols* indicate specific binding and *open symbols* indicate nonspecific binding. **B, Scatchard replot of saturation data.**

Figure 3.1

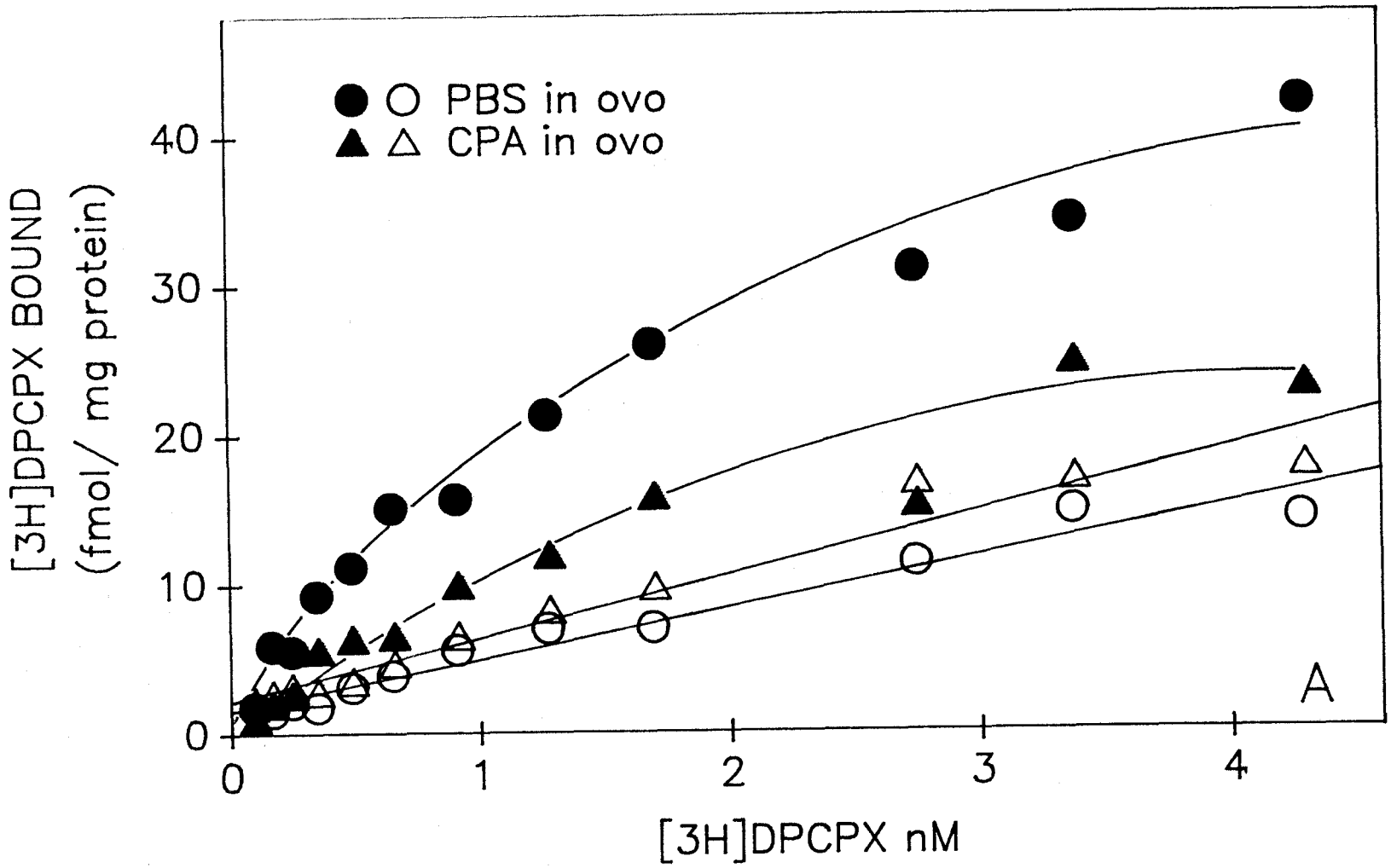
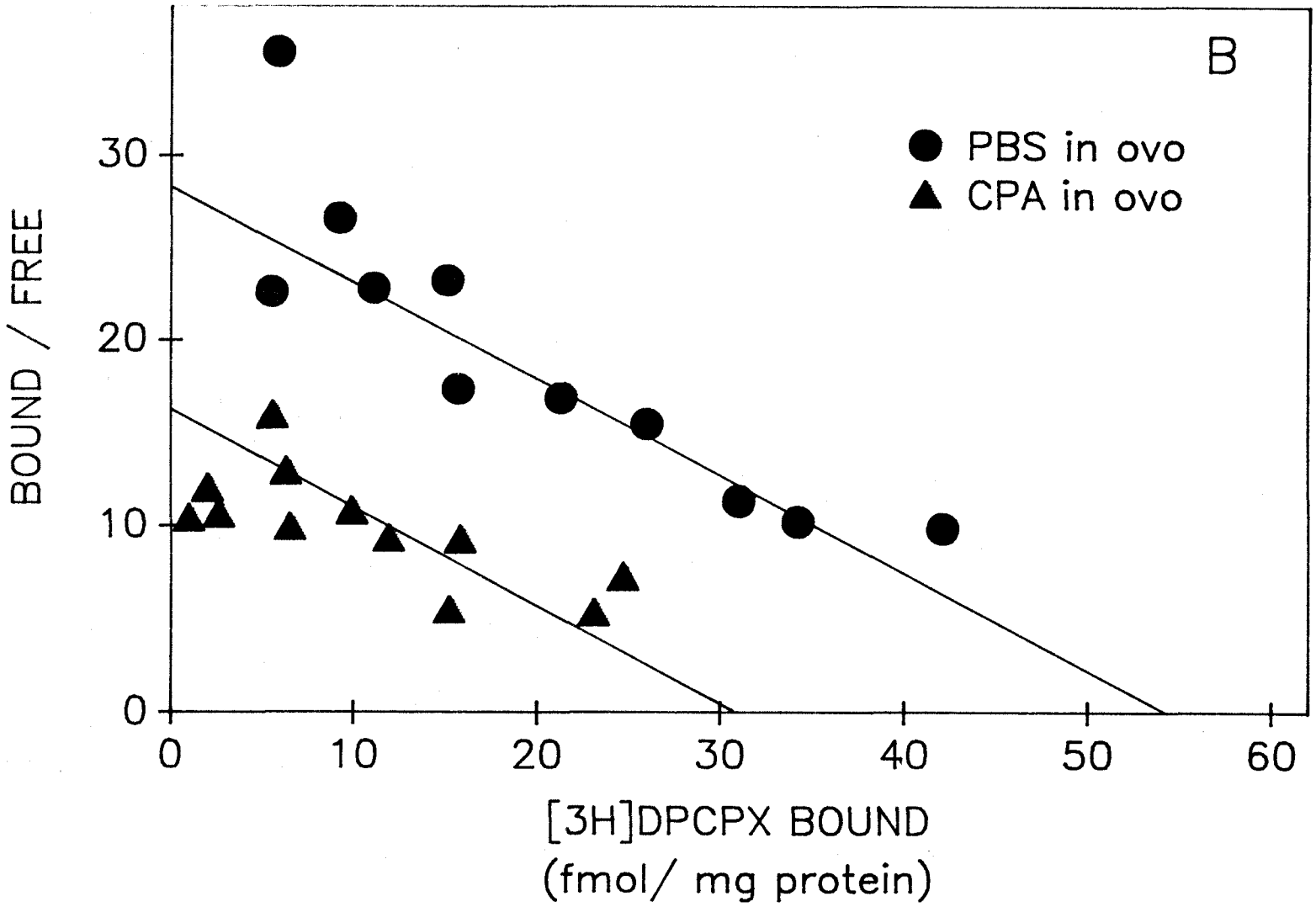

PBS in ovo
CPA in ovo
[3H]DPCPX BOUND
(fmol/ mg protein)
0
10
20
30
40
0
1
2
3
4
[3H]DPCPX nM
A

Figure 3.1


B
PBS in ovo
CPA in ovo
BOUND / FREE
0
10
20
30
0
10
20
30
40
50
60
[3H]DPCPX BOUND
(fmol/ mg protein)

a time-dependent decrease in the binding of [^{3}H]DPCPX to adenosine receptors in cardiac membranes (Fig. 3.2). A maximum decrease of 46% of the [^{3}H]DPCPX binding sites occurred at 8 hr after treatment and persisted for at least 24 hr, the longest time period evaluated.

To determine the dose dependency of the CPA-induced loss of A_1 receptors we treated day 9 embryos with increasing doses of CPA and assayed the cardiac membranes for [^{3}H]DPCPX binding sites after a 24 hour exposure period (Fig. 3.3). Administration of 0.1-10 lmoles of CPA *in ovo* led to graded reduction in the number of [^{3}H]DPCPX binding sites. The half maximal effect was obtained at a dose of 0.5 $\pm$ 0.02 lmol CPA with a maximal decrease in binding occurring at a dose of 3 lmol.

Attenuation of CPA-induced decrease in [^{3}H]DPCPX binding sites by theophylline

The co-injection of 10 lmol theophylline with 1 lmol CPA significantly reduced magnitude of the decrease in [^{3}H]DPCPX binding elicited by this dose of CPA. (Fig. 3.4). The 10 lmol dose of theophylline administered alone did not lead to a significant change in [^{3}H]DPCPX binding as compared to that of control membranes. These results suggest that the CPA induced loss of [^{3}H]DPCPX binding sites was a consequence of adenosine receptor activation.

Agonist binding to A_1 adenosine receptors

Agonist/antagonist competition curves were performed using unlabeled CPA and [^{3}H]DPCPX as an indirect means to characterize the

Figure 3.2. Time course of A_1 receptor decrease following agonist exposure. Chick embryos were treated *in ovo* with either PBS or 1 lmol CPA. At the indicated times, hearts were removed and assayed for [^{3}H]DPCPX specifically bound at a single, nonsaturating concentration (1.8 nM) of the radioligand. Values shown represent the average of 3-5 different membrane preparations with 6-12 hearts pooled per preparation.

Figure 3.2

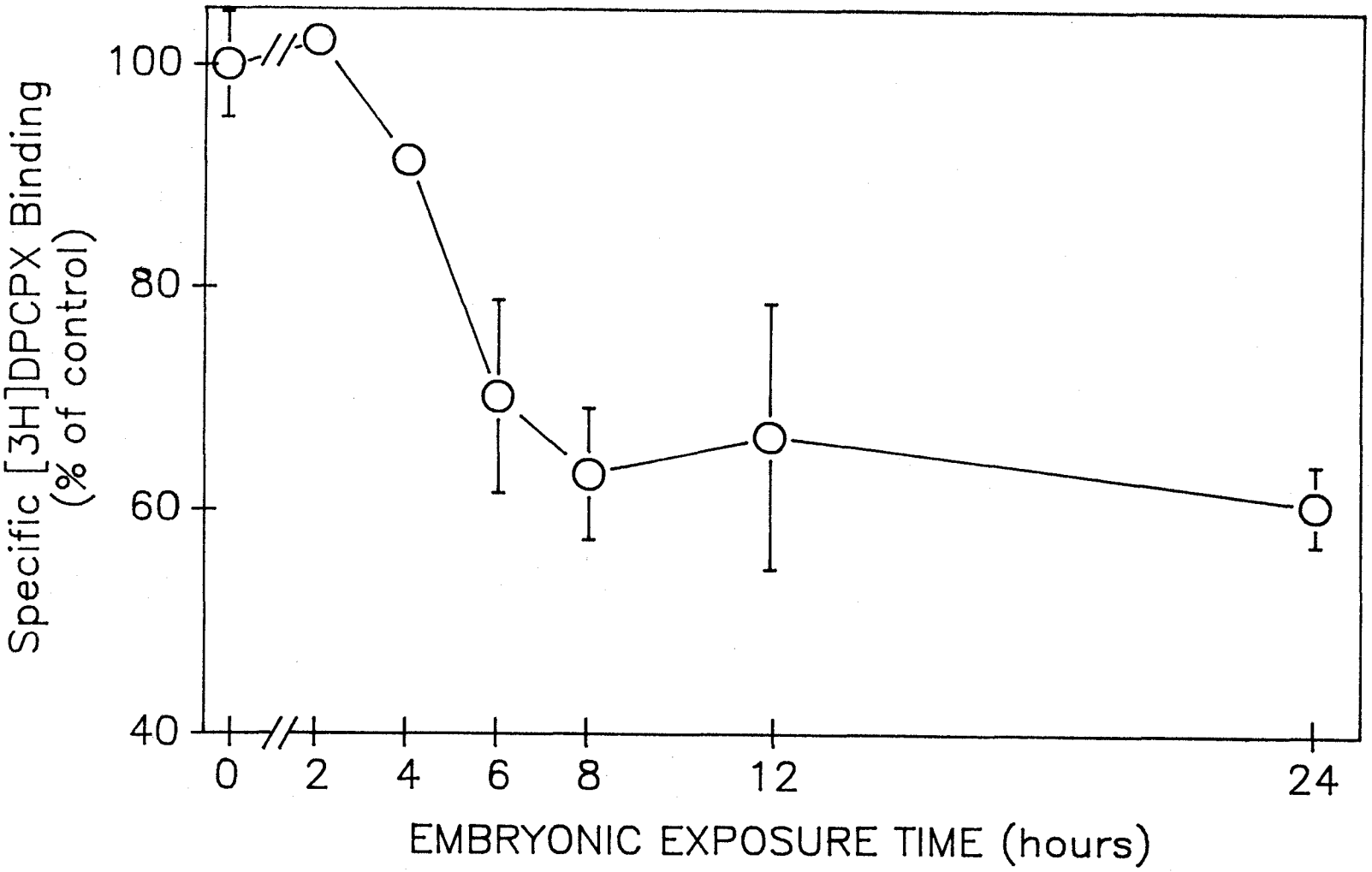

Specific [3H]DPCPX Binding
(% of control)
100
80
60
40
0
2
4
6
8
12
24
EMBRYONIC EXPOSURE TIME (hours)

Figure 3.3. Concentration-effect for cyclopentyladenosine induced A_1 receptor down regulation. Cardiac membranes derived from chick embryos treated for 24 hr with the dose of CPA shown were assayed for [^{3}H]DPCPX specifically bound at a single, nonsaturating concentration (1.9 nM) of the radioligand. The values are expressed as the % of [^{3}H]DPCPX bound in CPA-treated membranes compared to PBS treated membranes and represent the mean $\pm$ standard error of two membrane preparations, with 6 hearts pooled per preparation.

Figure 3.3

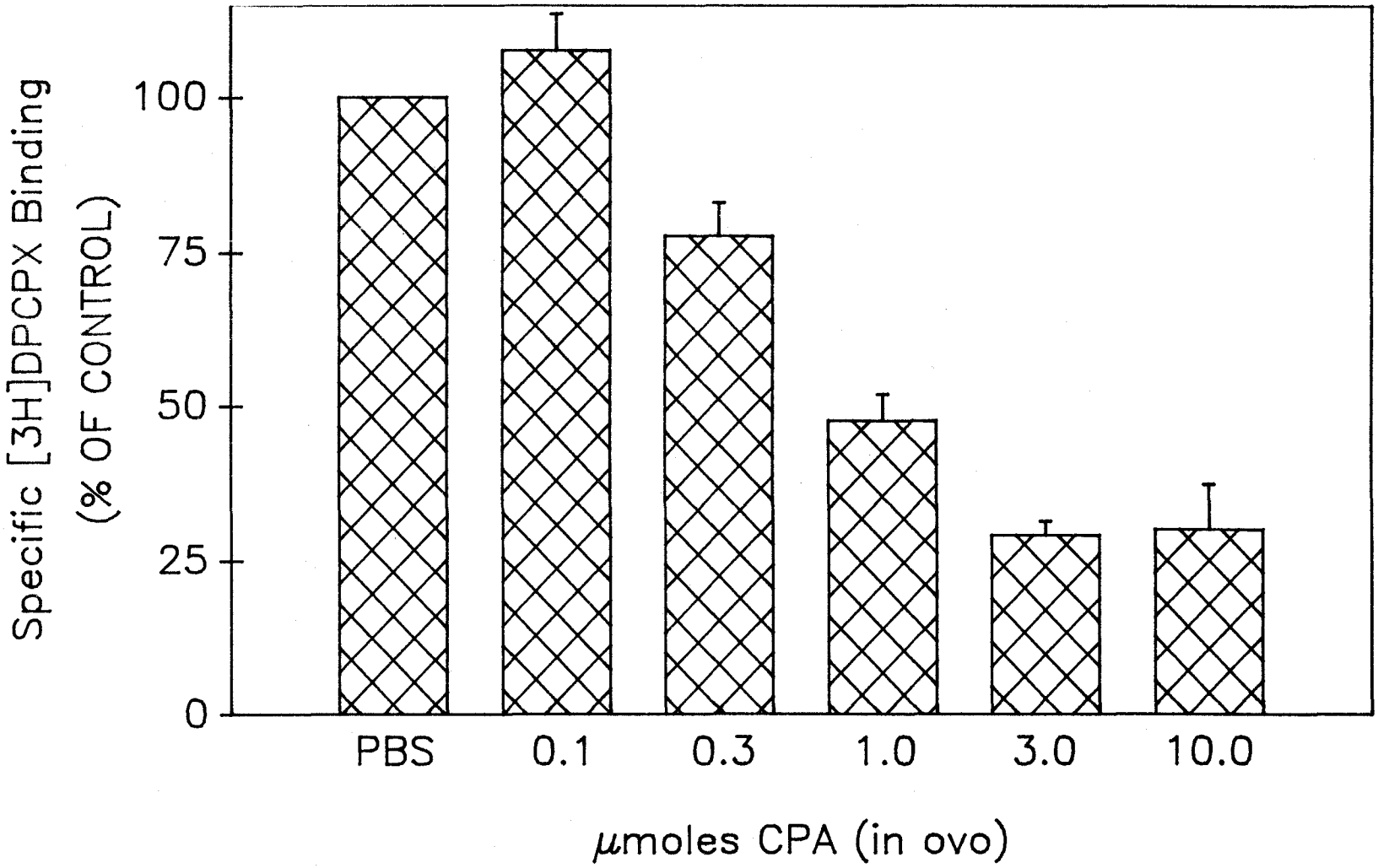
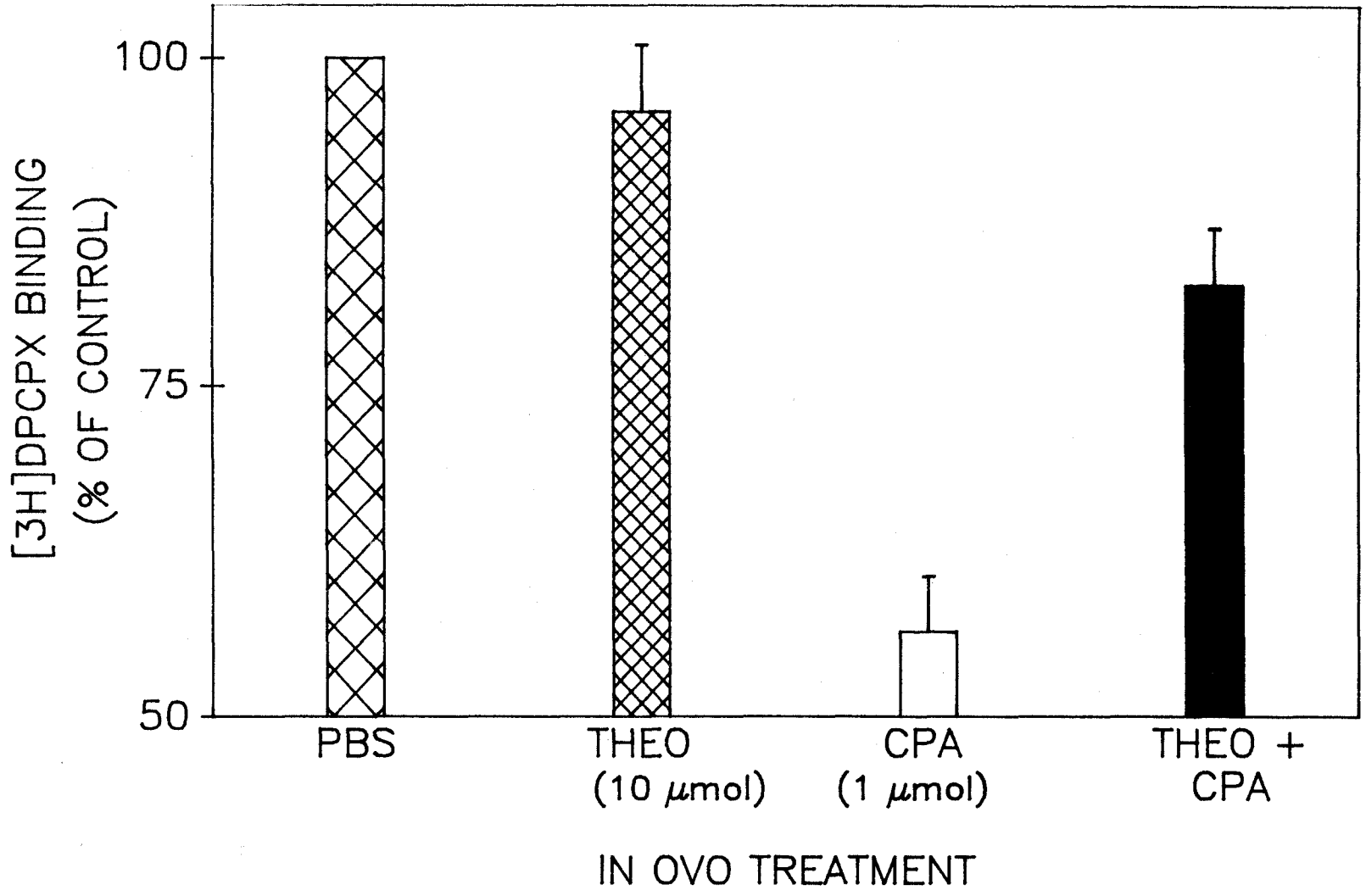

Specific [3H]DPCPX Binding
(% OF CONTROL)
100
75
50
25
0
PBS
0.1
0.3
1.0
3.0
10.0
μmoles CPA (in ovo)

Figure 3.4. **Effect of theophylline on the CPA-induced decrease in [^{3}H]DPCPX binding sites.** Embryos were exposed for 24 hr with either saline, 1 lmol CPA, 10 mmol theophylline or 1 lmol CPA co-injected with 10 mmol theophylline. Cardiac membranes were harvested at embryonic age day 10. Values depicted represent the mean $\pm$ standard error of duplicate experiments with 6 hearts pooled per experiment and are presented as the percentage of specific [^{3}H]DPCPX binding in cardiac membranes from each treatment group as compared to binding in membranes from saline treated embryos.

Figure 3.4


100
75
50
[3H]DPCPX BINDING
(% OF CONTROL)
PBS
THEO
(10 μmol)
CPA
(1 μmol)
THEO +
CPA
IN OVO TREATMENT

agonist binding parameters to A_1 adenosine receptors in cardiac membranes derived from saline- and CPA-treated embryos. Computer-assisted analysis of CPA competition data was used to quantify the high and low affinity constants (K_H and K_L respectively), as well as the fraction of receptors in each affinity state. The results depicted in Fig. 3.5 represent the simultaneous fit of data from 15 separate experiments. Under the assay conditions employed the distribution of A_1 receptors between high and low affinity states appeared to be similar in cardiac membranes for both treatment groups (Table 3.1). Moreover there were no significant difference in the high- and low-affinity equilibrium dissociation constants in the two treatment groups (Table 3.1). To further characterize A_1 adenosine receptor-G protein coupling in the two treatment groups, guanine nucleotide regulation of CPA binding was assessed. The addition of 10 uM GppNHp shifted the receptor population to a low affinity form and these data were adequately described by a one site model. A_1 receptors in cardiac membranes from both CPA and saline-treat embryos were therefore equally sensitive to guanine nucleotide modulation. These results suggest that the down regulation of A_1 receptors in response to *in ovo* CPA exposure is associated with a decrease in receptor density with no alteration in the distribution of the remaining receptors between the high and low affinity states.

CPA-induced decrease in [^{3}H]DPCPX binding sites as a function of development

Previous investigations utilizing embryonic chick atria have

Figure 3.5. CPA competition of [^{3}H]DPCPX specific binding to cardiac membranes derived from saline or CPA-treated embryos. Cardiac membranes were prepared and incubated in a fixed concentration of [^{3}H]DPCPX (1.4-2.1 nM) in the presence of increasing concentrations of CPA. Each point represents the mean $\pm$ standard error of 15 separate experiments. The competition curves were fitted using the iterative public procedure FITCOMP on the PROPHET computer with the data being best fit to a two-site model for receptor occupancy.

Figure 3.5

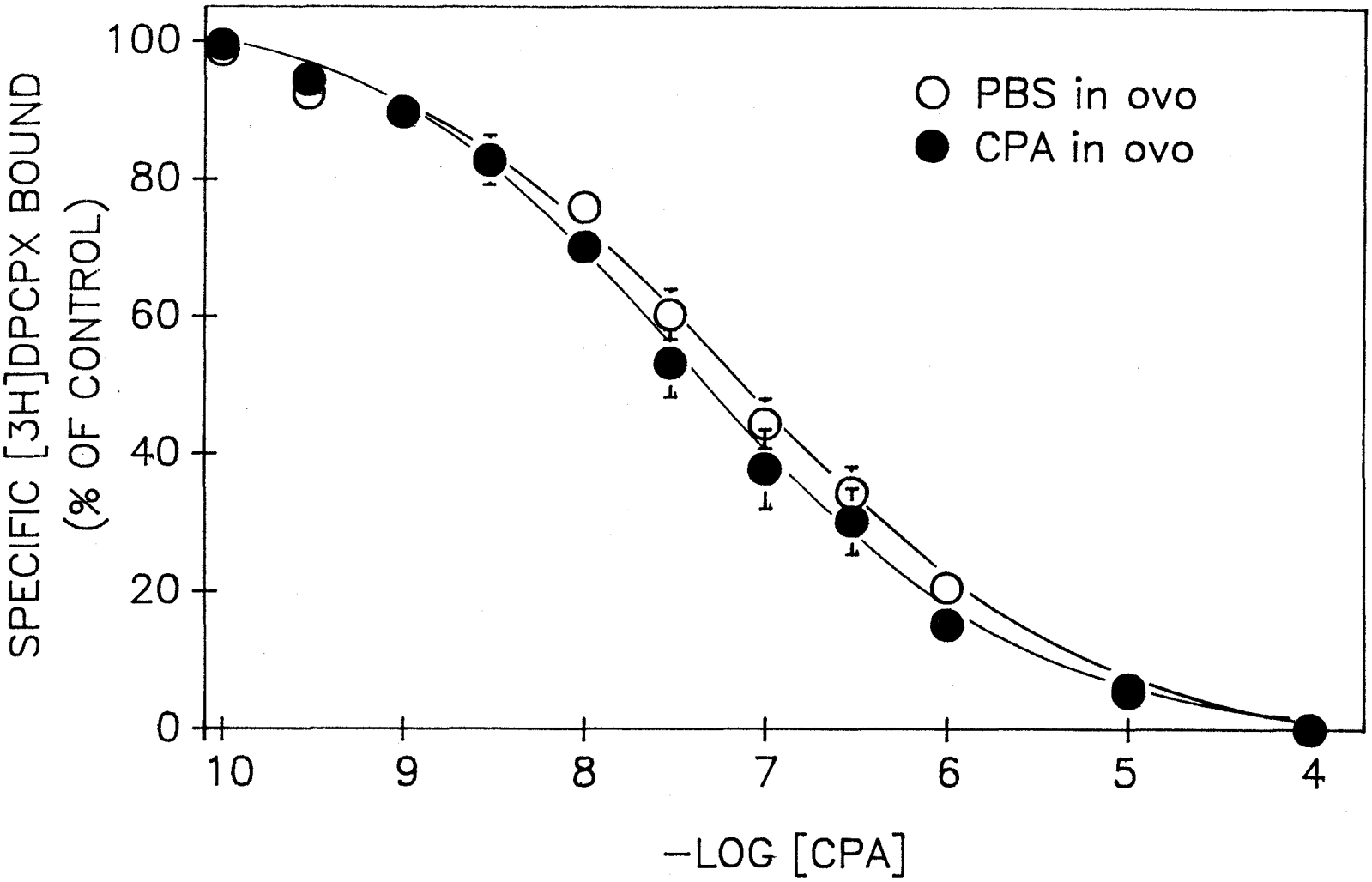

SPECIFIC [3H]DPCPX BOUND
(% OF CONTROL)
100
80
60
40
20
0
10
9
8
7
6
5
4
−LOG [CPA]
PBS in ovo
CPA in ovo

Table 3.1

Influence of *in ovo* treatment on CPA competition for [^{3}H]DPCPX binding to myocardial membranes.

In Ovo Treatment		K_H	R_H	K_L	R_L
		nM	%	nM	%
PBS	Control	0.69±0.92	23.9±8.9	202.2±51.4	76.1±4.4
	Gpp(NH)p	------	------	107.5±16.1	100.6±2.4
CPA	Control	0.58±0.22	23.0±12.0	118.0±36.1	77.0±6.0
	Gpp(NH)p	------	------	71.5±14.5	100.4±3.3

Values presented are the mean ± S.E.M. for 15 to 17 individual experiments. K_H and K_L are, respectively , the high and low affinity equilibrium dissociation constants, while % R_H and %R_L represent the percentage of receptors in either the high or low affinity states. The inhibition constants and percentages of receptors in a given state were determined using the iterative curve fitting routine LIGAND.

indicated that adenosine analogs elicit their negative chronotropic effect through activation of an A_1 receptor. The developmental profile for this response allowed us to ascertain the relative effectiveness of an adenosine agonist to down regulate A_1 receptors at both a physiologically unresponsive age (day 6) and at fully responsive ages (day 10 and 12). The results of these studies depicted in figure 3.6 indicate that there were no significant differences in CPA-induced down regulation of A_1 receptor density as a function of development. These data suggest that the functional coupling of the cardiac A_1 receptor to the negative chronotropic response is not required for the phenomenon of down regulation to be manifested.

Physiological responsiveness of isolated atria exposed to CPA in ovo

The effects of increasing concentrations of CPA on the spontaneous beating rate of isolated atria removed from embryos exposed to CPA *in ovo* at different stages of development are depicted in Fig. 3.7. Basal beating rates did not differ significantly between treatment groups at any of the ages evaluated. Atria isolated from CPA exposed embryos were, however, significantly less responsive to the CPA-induced negative chronotropic effect than the saline exposed control atria at each of the developmental stages examined. This change in sensitivity was primarily expressed as a decrease in the efficacy of CPA as a negative chronotrope. In addition there was a trend for CPA to be less potent in atria derived from embryos exposed to the agonist *in ovo* which was statistically significant in embryonic day 8 atria (Table 3.2). These results suggest that the down regulation of cardiac A_1

Table 3.2

Influence of embryonic age on the negative chronotropic response to CPA in atria from PBS- and CPA-treated embryos

	PBS		CPA	
Embryonic Age	E_{max}	EC_{50}	E_{max}	EC_{50}
days in ovo	%	1M	%	1M
8	63.8±1.7	0.08±0.01	55.0±1.8	0.68±0.09*
10	88.0±4.4	0.06±0.02	55.8±2.4*	0.18±0.04
12	103.6+4.0	0.09±0.02	72.8±3.1*	0.16±0.04

Values presented are the mean ± S.E.M. for 10 to 15 isolated atria in three separate experiments at each age characterized. E_{max} represents the maximum percent inhibition of beating and the EC_{50} value is the dose of CPA needed to elicit 50% of the maximal response.

*Statistically significant difference from the respective PBS-treated day using t-test for paired data ($P < 0.05$).

Figure 3.6 Developmental profile of CPA-induced down regulation of [^{3}H]DPCPX binding sites. Atrial membranes prepared from embryos treated *in ovo* with saline or CPA were assayed of the specific binding of [^{3}H]DPCPX at the ages indicated. Bar heights represent the percent of [^{3}H]DPCPX binding sites in CPA-treated cardiac membranes as compared to the [^{3}H]DPCPX binding sites in saline treated membranes. Each value is the mean $\pm$ standard error of 2 to 5 determinations for each embryonic age depicted.

Figure 3.6

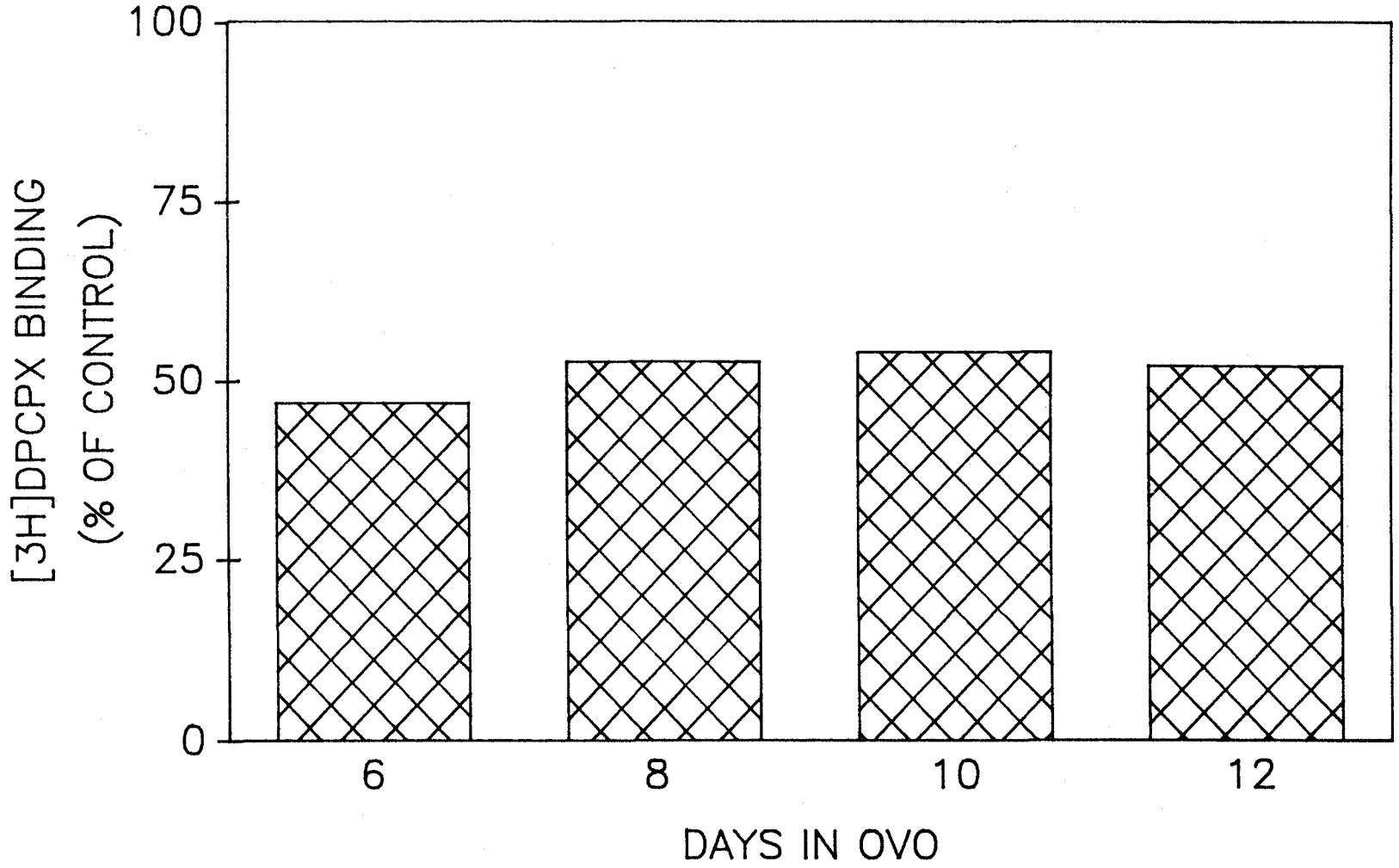
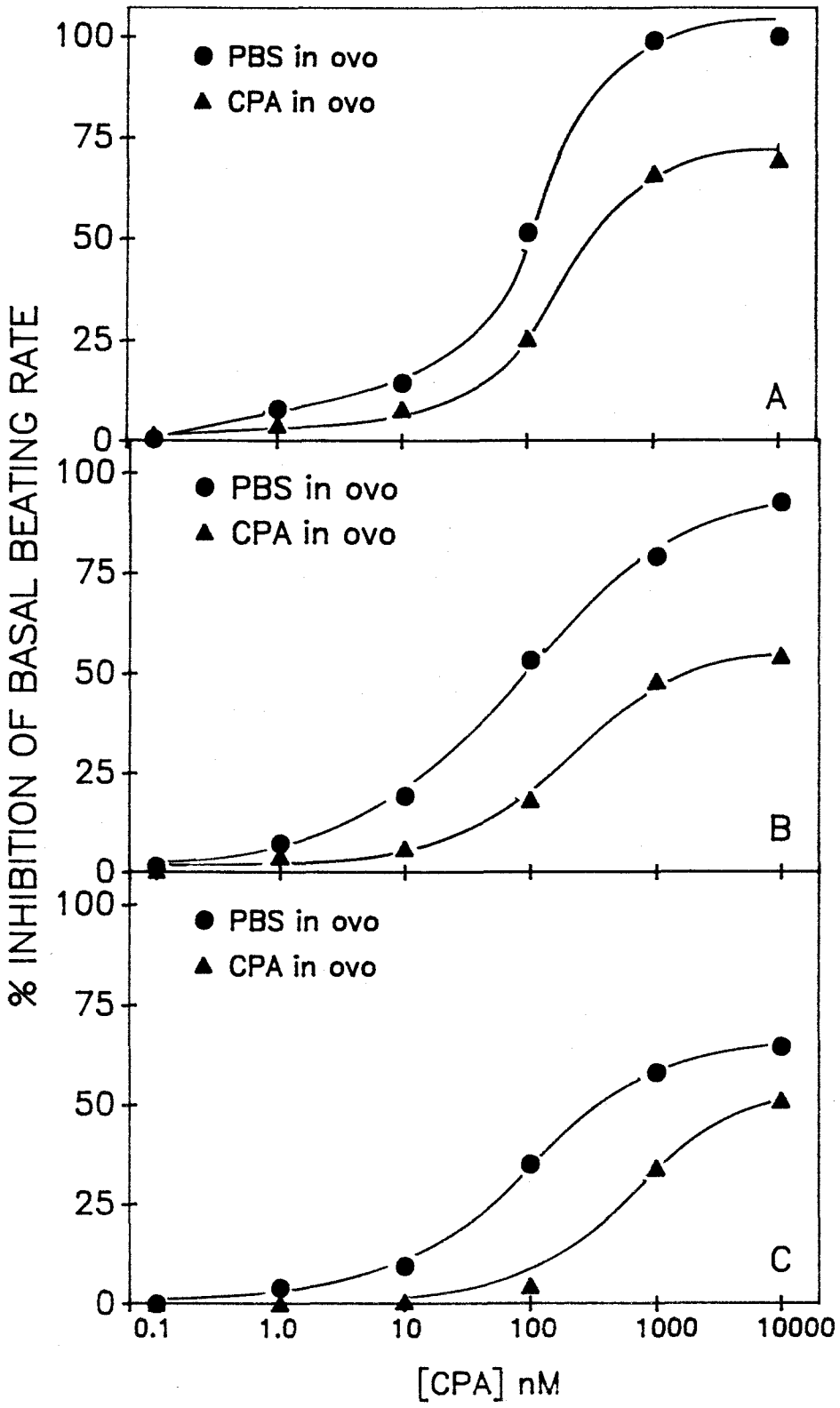


Figure 3.7. Concentration-response curves for CPA-induced negative chronotropic effect as a function of embryonic age. Concentration-response data represent the mean of percentage inhibition of spontaneous beating rate in atria from CPA- or saline-treated embryos from embryonic ages day 12 (A), 10(B), and 8 (C). Values represent data from 8 to 12 atria for each treatment at each age.

Figure 3.7


% INHIBITION OF BASAL BEATING RATE
100
75
50
25
0
PBS in ovo
CPA in ovo
A
B
C
0.1
1.0
10
100
1000
10000
[CPA] nM

adenosine receptors in embryonic chick heart is accompanied by a reduced responsiveness to the negative chronotropic effects of CPA.

Effect of *in ovo* treatment on CPA-induced decreases in cAMP content as a function of development

As a biochemical measure of A_1 adenosine receptor function, the ability of CPA to decrease cAMP content in isolated whole hearts was investigated. The beating rate assays showed a concentration of 10 1M CPA was able to effect a maximal decrease of spontaneous beating in isolated atria and was therefore the concentration used in these studies. Basal cAMP content did not differ significantly between hearts removed from PBS- and CPA-treated embryos at 6, 9 or 12 days (Table 3.3). The addition of 10 1M CPA in vitro decreased cAMP levels in hearts removed from day 9 and 12 embryos in both treatment groups. While there was a trend for CPA to decrease cAMP content to a greater extent in hearts from PBS-treated embryos, these differences were not statistically significant. In contrast, this concentration of CPA did not affect the cAMP content of day 6 embryonic hearts in either treatment group. These results suggest that down regulation of cardiac A_1 adenosine receptors did not alter the in vitro responsiveness of adenylyl cyclase to negative modulation by CPA.

E. DISCUSSION

A wide variety of pharmacological and physiological conditions have been reported to alter the efficiency with which adenosine receptor activation effects a biological response. Thyroid hormone

Table 3.3

Influence of embryonic age on CPA mediated decrease in cyclic AMP content in isolated whole hearts.

Embryonic Age	*In Ovo* Treatment	Cyclic cAMP Content Basal	CPA (10 1M)	Percentage Decrease
(Day)		pmol/mg protein		
6	PBS	9.5±2.8*	10.3±2.4	------
	CPA	8.4±2.5*	9.9±2.5	------
9	PBS	17.9±0.2	10.8±0.3	42.9±0.3
	CPA	18.4±1.7	12.4±0.7	30.7±8.4
12	PBS	21.0±1.9	15.6±2.4	27.8±4.9
	CPA	18.0±3.2	16.2±2.8	18.0±4.0

Cyclic AMP content was measured in hearts prepared from embryos of the indicated age as described in the Methods section. Values presented are the mean ± S.E.M. for 2-4 separate experiments for each age. The % decrease represents the decrease in cAMP content in the presence of 10 1M CPA as compared to cAMP content under basal conditions.
*Statistically significant difference from basal levels of other ages using Student's t-test ($P < 0.01$).

status, pregnancy, aging, anoxia and chronic exposure to xanthines all appear to alter the sensitivity of tissues to adenosine or its analogs (Bumgarner et al., 1989; Lee et al., 1986; Ramkumar et al., 1988; Sanders et al., 1988). The developing chick embryo provides a useful model system to evaluate the effects of persistent agonist activation of cardiac A_1 adenosine receptors. We have demonstrated that sustained exposure *in ovo* to an A_1-selective agonist leads to a time-and dose-dependent decrease in cardiac A_1 adenosine receptor density. . The loss of A_1 adenosine receptors was attenuated by co-injection of the adenosine receptor antagonist theophylline. These results are consistent with the involvement of a receptor-mediated mechanism in the observed down regulation of cardiac adenosine receptors. A functional correlate of the reduction in A_1 receptor density was a decreased physiological responsiveness of isolated atria to CPA-induced negative chronotropy.

Several recent studies have reported agonist induced decreases in A_1 adenosine receptors as detected with agonist radioligands in a variety of tissues, including adipocyte preparations (Hoffman et al.,1986; Parsons et al., 1987), and embryonic chick heart (Shryock et al., 1989). We have extended these findings by using an antagonist radioligand to differentiate a decrease in the density of A_1 adenosine receptors from changes in the agonist affinity states of the receptor. Antagonists do not discriminate between agonist affinity states of the receptor allowing a quantification of receptor specific activity irrespective of alterations in receptor-G protein coupling. The decrease in [^{3}H]DPCPX binding in cardiac membranes following *in ovo*

exposure to CPA was shown to be a consequence of a decrease in [^{3}H]DPCPX receptor density.

The effects of *in ovo* exposure to CPA were examined at several stages of embryonic development to permit an assessment of the relationship between physiological responsiveness and sensitivity to CPA-induced down regulation of A_1 adenosine receptors. At embryonic day 6, isolated atria are insensitive to the negative chronotropic effects of CPA (Blair et al., 1989). In contrast, the magnitude of the down regulation of A_1 receptors at this age was comparable to that observed in atria isolated from embryos displaying maximum physiological responsiveness (Fig. 3.6). These data imply that functional coupling to this effector system is not a necessary prerequisite for agonist induced down regulation of A_1 receptors.

The sensitivity of the *in ovo* exposed atria to CPA-induced negative chronotropy was evaluated in an attempt to establish a physiological correlate of the down regulation. At embryonic day 8, a stage of development at which isolated atria are not fully responsive to CPA-induced inhibition of spontaneous beating, down regulation of A_1 adenosine receptors was accompanied by a decrease in the potency and efficacy of the adenosine analog. In atria derived from embryos at more responsive stages of development, down regulation was associated only with a significant decrease in the efficacy of CPA as a negative chronotrope. These data are in accordance with earlier studies in which we demonstrated that a large fractional occupancy of A_1 adenosine receptors is required to express negative chronotropy during this period of embryonic development (Blair et al., 1989). The decrease in

both the potency and efficacy of CPA in exposed embryonic day 8 atria may indicate that the fractional occupancy required for maximum physiological response in day 8 atria is less than that necessary in embryonic day 10 and 12 atria. These results are also in agreement with those recently reported by Shryock et al. (1989) who demonstrated that down regulation of chick heart A_1 adenosine receptors was accompanied by a decrease in the efficacy of R-PIA as a negative inotropic agent.

A_1 receptors have been shown to be coupled to the inhibition of adenylyl cyclase in embryonic chick heart (Blair, et al., 1989). However, basal concentrations of cAMP were similar in hearts from both saline- and CPA-treated embryos which is in contrast to the response reported for adipocytes chronically exposed to an adenosine agonist. A_1 adenosine receptors coupled to the inhibition of adenylyl cyclase in adipocytes have been shown to exhibit both a down regulation of agonist radioligand binding sites and an increase in basal and stimulated adenylyl cyclase activity following chronic exposure to adenosine analogs (Hoffman et al., 1986; Green, 1987; Parsons and Stiles, 1987). A similar dual response has been reported for embryonic chick primary cardiac myocyte cultures following chronic exposure to muscarinic agonists (Linden, 1987). In accordance with the results of Shryock et al. (1989), we did not observe an adaptive increase in cAMP concentration in cardiac tissue subsequent to *in ovo* exposure to an adenosine agonist. Thus, the phenomenon of increased adenylyl cyclase activity as a consequence of chronic exposure to agonists may require a higher receptor density than that associated with the level of expression of A_1 receptors in cardiac tissue.

The ability of an adenosine agonist to elicit a decrease in cAMP content in cardiac tissue was found to be similar in control hearts and in hearts in which the receptor density had been reduced by approximately 46%. These data are congruent with a previous report indicating the presence of spare receptors for A_1 receptor regulation of adenylyl cyclase in cardiac tissue (Blair et al., 1989). Blair et al. (1989) also reported that A_1 receptors are coupled to the inhibition of adenylyl cyclase as early as embryonic day 5. In contrast, we were unable to detect a CPA-induced decrease in cAMP content in embryonic day 6 hearts. Although CPA is a selective A_1 receptor agonist, high concentrations of CPA (>1 1M) may be sufficient to activate the stimulatory A_2 receptor. In the present studies whole heart preparations were employed which possess several different cell types. In day 6 hearts the ratio of A_1 to A_2 receptors may be unfavorable for the detection of A_1 receptor mediated inhibition of adenylyl cyclase inasmuch as concomitant activation of the A_2 receptors in smooth muscle and endothelial cells would elevate cAMP levels. In previous studies CPA-mediated inhibition of adenylyl cyclase activity was demonstrated in membranes prepared from cardiac tissue in which steps had been taken to minimize the content of vascular components and thus reduce the A_2 mediated stimulatory response (Blair et al., 1989).

Agonist/antagonist competition experiments were performed to assess the distribution of receptors in the high and low affinity states of the cardiac A_1 adenosine receptor. The results of these studies indicated that CPA recognizes two affinity states of the receptor in both control and treated membranes. The [^{3}H]DPCPX/CPA

competition data demonstrated that *in ovo* exposure to CPA did not affect A_1 receptor-G protein coupling. Both the high and low affinity dissociation constants and the fraction of receptors in each affinity state were unchanged following A_1 receptor down regulation. This is in contrast to what has been found for the muscarinic receptor of embryonic chick heart under similar experimental conditions. The cardiac muscarinic receptor system exhibits many similarities to cardiac adenosine receptors with respect to their transduction mechanisms, effector systems and receptor regulation (Halvorsen et al., 1981, Liang et al.,1986, Kurachi et al., 1986). However, Halvorsen et al.(1981) have reported that the down regulation of [H]QNB binding sites following *in ovo* exposure to muscarinic agonists was manifested as a preferential decrease in the high affinity state. This difference may be related to the tightness of coupling of A_1 adenosine receptors to G proteins relative to the coupling of muscarinic receptors to G proteins in cardiac tissue. Agonist binding to each of these receptors is subject to negative modulation by guanine nucleotides, however muscarinic receptors in cardiac membranes have demonstrated a high affinity state for agonists that is quantitatively converted to a low affinity state in the presence of low concentrations of guanine nucleotides (Berrie et al., 1979). In contrast, cardiac A_1 adenosine receptors in membrane preparations required significantly higher concentrations of guanine nucleotides to shift high- to low-affinity states and in most studies, only a partial transition from the high affinity into the low affinity state was accomplished (Lohse et al., 1985; Martens et al., 1987; Leid et al., 1988; Stroher et al., 1989).

In summary, activity-dependent down regulation of A_1 adenosine receptors has been demonstrated in embryonic chick hearts. This down regulation was not associated with an uncoupling of the A_1 receptor from G proteins. Moreover the reduced sensitivity to CPA-induced negative chronotropy in treated atria was observed to represent a functional correlate of the A_1 adenosine receptor down regulation. The ability to investigate A_1 receptor regulation in a preparation conducive to chronic *in ovo* exposure suggests that the embryonic chick heart represents a useful model system in which to study the mechanisms involved in the regulation of cardiac adenosine receptors.

CHAPTER 4

SUMMARY

The study of ontogeny has been widely used as a tool to characterize receptor systems associated with the autonomic innervation of the embryonic chick heart. The developing chick heart is particularly well suited for this type of study because gestational age can be determined precisely allowing temporal correlation of key developmental events. We have employed this method of investigation to study the cardiac adenosine receptor system and its relationship to an adenosine mediated negative chronotropy in embryonic chick heart.

Myocardial responsiveness to adenosine stimulation is presumed to be mediated by a complex series of interactions set in motion at the receptor site subsequent to agonist binding. A characterization of the developmental profile for the adenosine analog mediated negative chronotropic response in isolated embryonic chick atria revealed the onset of physiological sensitivity to adenosine analogs, as evidenced by a decrease in spontaneous beating rate, began at embryonic day 7 and gradually increased through day 12 at which time the atria were fully responsive. The competitive antagonism of the response to 2-chloroadenosine by an antagonist impermeant to cell membranes and the rank order potency for the adenosine analogs as negative chronotropes support the role of a membrane-bound A_1 adenosine receptor in this

physiological response.

Temporal correlation of the development for the adenosine receptor mediated negative chronotropy with changes in the density of receptor binding sites was assessed with radioligand binding studies in cardiac membranes derived from key developmental stages. The availability of a radioactively labeled antagonist ligand, [3H]DPCPX, that selectively binds to A_1 adenosine receptors with high affinity and possesses a high specific activity, allowed us to characterize adenosine receptors in a tissue with a low density of adenosine binding sites. Equilibrium saturation binding of [3H]DPCPX revealed the presence of adenosine receptors early in embryonic development, prior to the onset of physiological sensitivity to adenosine analogs. An increase in receptor density occurred at a stage of development that was temporally correlated with an increase in atrial responsiveness to the negative chronotropic effects of adenosine receptor agonists. These results suggest that a large fractional occupancy of A_1 adenosine receptors is required to express negative chronotropy during this period of embryonic development.

Characterization of the binding properties of the A_1 adenosine receptors in membranes derived from unresponsive and fully responsive hearts revealed two affinity states for agonists in the absence of added guanine nucleotides. Membranes from hearts at unresponsive ages demonstrated a higher percentage of the total receptor population in the high affinity state as compared with hearts from fully responsive stages of development. The development of physiological sensitivity was associated with a reduced coupling between the receptor and G proteins

as evidenced by a shift from high affinity sites to low affinity sites and may be an expression of a desensitization phenomenon.

A_1 adenosine receptors have been recently characterized in human atrial and ventricular myocardium with the antagonist radioligand [3H]DPCPX allowing evaluation of adenosine receptor-G protein coupling for the first time in this tissue (Bohm, et al., 1989). The rank order of potency for agonists and antagonists in physiological and radioligand binding studies were characteristic for an A_1 adenosine receptor subtype. Agonist/antagonist competition studies revealed high and low affinity states for agonists that were responsive to negative modulation of binding by guanine nucleotides. The data presented by Bohm et al., (1989) indicates the human cardiac adenosine receptor is very similar in its binding parameters to the embryonic chick cardiac A_1 adenosine receptor we have described. This suggests use of the chick heart is relevant to the understanding of human adenosine receptors.

Characterization of the sensitivity of adenylyl cyclase to inhibition by CPA during embryogenesis revealed that A_1 adenosine receptors were functionally coupled to adenylyl cyclase at both unresponsive and fully responsive ages. The efficacy of CPA as an inhibitor of adenylyl cyclase activity was comparable at all stages of development evaluated including the developmental period when A_1 receptor density increased approximately 2.5 fold. This data supports the presence of spare receptors in this adenosine receptor mediated biochemical response. Because the functional coupling of the adenosine receptor to adenylyl cyclase occurs early in embryonic development, this event is not temporally correlated with the onset of physiological

sensitivity. This 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 argue against a role for adenylyl cyclase in mediating a negative chronotropic response. Recent evidence of cardiac adenosine receptors coupling to a potassium channel independent of soluble second messengers presents a more plausible molecular mechanism for the adenosine receptor mediated negative chronotropy (Kurachi et al., 1986). However this leaves unanswered the question of what the role of adenosine receptor mediated inhibition of basal adenylyl cyclase is in the myocardium. The ability of adenosine receptors to mediate inhibition of basal adenylyl cyclase has been questioned, however the majority of studies, including the data presented here, indicates A_1 adenosine receptors can effect an inhibition of adenylyl cyclase in the absence of previous stimulation of activity.

Several possible roles for inhibition of basal activity have been suggested. Earlier studies have suggested that inhibition of basal adenylyl cyclase results in a direct negative inotropic effect in atrial tissue (Rockoff and Dobson, 1980; Evans et al., 1982). However this effect may also be attributed to the activation of an inwardly rectifying K^+ channel resulting in a shortened action potential and therefore reduced influx of calcium. Another possible role for adenosine receptors in the cardiovascular system has recently been suggested by Adair et al. (1989). These authors have presented data indicating that adenosine has a physiological role in receptor-mediated growth regulation of the vascular system in the chick embryo. The

angiogenic properties of adenosine have been previously described for *in vitro* preparations although the role of cAMP or the subtype of adenosine receptor involved in angiogenesis has not been investigated in either experimental situation (Meininger et al., 1986, Ziada et al., 1984). However the adenosine receptor identified in the vasculature is of the A_2 subtype which is associated with the stimulation of adenylyl cyclase. The atrial A_1 adenosine receptor may therefore have a function opposing angiogenesis in the myocardium. Xenophontos et al. (1989) have recently demonstrated increased rates of protein synthesis following elevation of cyclic AMP levels in rat heart. Although the effects of adenosine on this response were not evaluated, a muscarinic agonist blocked the increase in cAMP levels and prevented the increase in protein synthesis occurring in response to adenylyl cyclase stimulation. It would be interesting to investigate the possibility of a modulatory role for adenosine in protein synthesis under basal conditions.

Down regulation of the embryonic chick cardiac adenosine receptor was demonstrated following sustained *in ovo* exposure to an adenosine receptor agonist. The involvement of a receptor mediated mechanism in the agonist induced down regulation is supported by an attenuation of the response with an adenosine receptor antagonist and the time- and dose-dependent characteristics of the response. The use of an antagonist radioligand allowed us to determine that the decrease in [3H]DPCPX binding sites in cardiac membranes following *in ovo* exposure to CPA was a consequence of a decrease in receptor density and not a change in affinity of the receptor for the radioligand.

We determined the developmental profile for the agonist induced down regulation of A_1 adenosine receptors. This revealed that the response obtained in atria isolated from embryos insensitive to the negative chronotropic effects of CPA was comparable to the response observed in atria isolated from embryos displaying maximum physiological responsiveness. These data imply that functional coupling to this physiological response is not a necessary prerequisite for agonist-induced down regulation of A_1 receptors.

Down regulation of the cardiac A_1 adenosine receptor with *in ovo* exposure to CPA was accompanied by a decrease in the potency and efficacy of an adenosine analog to elicit a negative chronotropic response. These data are in accordance with our findings that a large fractional occupancy of A_1 adenosine receptors is required to express negative chronotropy. This suggests the possibility that under normal physiological conditions cardiac adenosine receptors may exist in a partially down regulated state. Data presented by Wu et al. (1989) demonstrated the up regulation of cardiac A_1 adenosine receptors in guinea pig heart following chronic *in vivo* treatment with the adenosine receptor antagonist theophylline supports this hypothesis. The up regulation of receptors was accompanied by an enhanced sensitivity of ventricular myocytes to both the electrophysiological and biochemical actions of adenosine receptor agonists. These studies imply that the level of endogenous adenosine is sufficiently high under normoxic conditions to partially occupy and down regulate cardiac adenosine receptors.

The ability of CPA to mediate a decrease in cAMP content following

down regulation was evaluated at several stages of development. Basal concentrations of cAMP were similar in hearts from saline and CPA-treated embryos. This suggests the cardiac adenosine receptor system in embryonic chick heart does not respond with an increase in basal and stimulated adenylyl cyclase activity as demonstrated in other tissues (Hoffman et al., 1986, Parsons and Stiles, 1987). CPA elicited a decrease in cAMP levels in hearts from both saline- and CPA-treated embryos supporting the presence of spare receptors in the coupling of the A_1 receptor to this biochemical response. However, hearts removed from a physiologically insensitive stage of development were also insensitive to the effects of CPA to elicit a decrease in cAMP levels. This may be the result of the experimental conditions employed in which the A_1 adenosine receptor mediated inhibition of adenylyl cyclase is masked by the concomitant activation of A_2 adenosine receptors found in the coronary vasculature.

The results of our studies indicate the embryonic chick A_1 adenosine receptor in cardiac membranes exhibits both high affinity and low affinity states for agonists. [3H]DPCPX/CPA competition data demonstrated that in ovo exposure to CPA did not affect either the high or low affinity dissociation constants and the fraction of receptors in each affinity state. Negative modulation of agonist binding by guanine nucleotides was similar in membranes from both treatment groups. These findings suggest that down regulation was not associated with an uncoupling of the A_1 adenosine receptor from G proteins.

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