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STIMULATION OF PHOSPHOINOSITIDE HYDROLYSIS AND INHIBITION OF CYCLIC AMP FORMATION BY MUSCARINIC AGONISTS IN DEVELOPING CHICK HEART*

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Abstract—As early as 4 days *in ovo*, phosphoinositide hydrolysis in embryonic chick heart was stimulated by the cholinergic agonist carbachol. In the 4-day chick heart, the concentrations of carbachol giving half-maximal and maximal stimulation of [^{3}H]inositol 1-phosphate formation were 30 μM and 1 mM respectively. These values are identical to those obtained using hearts from 13-day embryos [J. H. Brown and S. L. Brown, *J. biol. Chem.* **259**, 3777 (1984)]. The amounts of [^{3}H]inositol 1-phosphate formed per mg protein in the presence of carbachol were greater at early ages than at later ones; the stimulation by carbachol thus decreased from 8-fold at 4 days to 2-fold at 13 days. Muscarinic receptor stimulation also led to inhibition of isoproterenol-stimulated cyclic AMP formation as early as 4 days. The isoproterenol-stimulated increase in cyclic AMP was greatest at early ages, whereas the inhibitory effect of carbachol remained constant at 75%. At 4 days, half-maximal and maximal inhibition by carbachol occurred at 0.3 and 30 μM, respectively, the same values obtained using 13-day hearts (see reference cited above). Thus, the two biochemical responses of embryonic chick heart to muscarinic agonists have identical agonist sensitivities regardless of embryonic age and are functional prior to parasympathetic innervation and physiological responsiveness.

The development of parasympathetic innervation, neuroeffector transmission, and physiological responsiveness in the embryonic chick heart has been described extensively in the classic review by Pappano [1]. Information from this review and from muscarinic receptor binding studies [2, 3] exposes the problem addressed by this investigation: physiologically inactive muscarinic receptors exist prior to the onset of functional responsiveness. Briefly, muscarinic receptors are present in embryonic chick heart as early as 3 days *in ovo* [2], and vagal innervation occurs after day 4 [4]. Although innervation and receptors are present at these early stages, acetylcholine does not effect changes in heart rate until 5 days, and this response does not reach adult levels until 7 days [3].

The sequence of molecular changes by which acetylcholine regulates heart rate is not known. A number of cellular events occur in response to muscarinic agonists, including inhibition of catecholamine-stimulated adenylate cyclase activity [5–8] and stimulation of phosphoinositide turnover [9–11]. To determine if the failure of receptors to effect a physiological response could reflect delayed development of biochemical events that couple muscarinic receptors to the physiological response, we examined phosphoinositide turnover and cyclic AMP formation in embryonic chick hearts of various ages. Characteristics of the two responses in 13-day chick embryo hearts were described recently by this laboratory [11]. This is the first report describing the development of these two responses to muscarinic agonists in embryonic chick heart.

MATERIALS AND METHODS

Fertilized White Leghorn eggs were incubated at 39° in a Leahy 1200 incubator. Stages of development were determined according to Hamburger and Hamilton [12]. Hearts were dissected out and trimmed free of large vessels. Those 6 days and older were cut into pieces. Tissue was kept on ice or in oxygenated Kreb's buffer with glucose (118 mM NaCl, 4.7 mM KCl, 3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.5 mM EDTA, 10 mM glucose, pH 7.4) until all the tissue was collected. Hearts held in warm buffer were found to have higher rates of [^{3}H]Ins1P‡ formation than those held on ice; therefore, except for the experiment shown in Fig. 1, all tissue was kept warm and oxygenated prior to use.

Phosphoinositide response measurements. Phosphoinositide breakdown was quantitated by measuring the accumulation of the hydrolysis product [^{3}H] Ins1P in the presence of 10 mM LiCl, as described previously [10]. Depending on age, duplicate measurements were made using tissue from one to nine hearts. The samples were preincubated at 37° for 1 hr in Kreb's buffer containing glucose and 8 μCi/ml *myo*-[2-^{3}H]inositol (sp. act. 15.8 Ci/mmole). The 0.5 ml volume was oxygenated with 95% O_2/5% CO_2

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‡ Abbreviations: Ins1P, *myo*-inositol 1-phosphate; CARB, carbachol; ISO, isoproterenol; IBMX, isobutylmethylxanthine; and TCA, trichloroacetic acid.

gas. Carbachol and 10 mM LiCl were added for 30 min. The incubation was stopped when the medium was removed and the tissue rinsed with cold saline followed immediately by addition of ice-cold MeOH or by freezing in Freon and liquid N_2 until extraction was performed. The water-soluble [^{3}H]-Ins1P was extracted and separated from [^{3}H]inositol by anion exchange chromatography as previously described [10]. Briefly, samples were homogenized in cold $CHCl_3$:MeOH:H_2O (1:2:0.8), and a two-phase system was achieved with the addition of $CHCl_3$ and H_2O, making the solution $CHCl_3$:MeOH:H_2O (2:2:1). The aqueous upper phase was applied to an anion exchange resin, and [^{3}H]Ins1P was eluted with 8 ml of 200 mM ammonium formate/100 mM formic acid. Eluents were combined with an equal volume of Liquiscint (National Diagnostics) and assayed for radioactivity by liquid scintillation counting. Parallel samples were assayed for protein according to the method of Bradford [13].

Cyclic AMP measurements. The muscarinic response was quantitated by determining the ability of carbachol to inhibit isoproterenol-stimulated cyclic AMP generation. Hearts of various ages were preincubated at 37° for 30 min in Kreb's buffer with glucose and 0.1 mM isobutylmethylxanthine. One to five hearts constituted a sample, and samples were done in duplicate. All drugs were added for 2.5 min in the presence of 0.1 mM ascorbate. Incubations were terminated by homogenizing the tissue in 0.5 ml of ice-cold 10% trichloroacetic acid. The TCA-insoluble material was analyzed for protein according to the method of Bradford [13]. Cyclic AMP in the TCA-soluble fraction was purified and assayed as described previously [14], by the competitive protein binding assay of Gilman [15]. When inhibition of isoproterenol-stimulated cyclic AMP formation is expressed as a percentage, the calculation is as follows:

$$\% \text{ Inhibition} = \left\{1 - \left(\frac{(\text{ISO} + \text{CARB}) - (\text{CARB})}{(\text{ISO}) - (\text{Control})}\right)\right\} \times 100$$

Materials. [^{3}H]Inositol was obtained from the New England Nuclear Corp., Boston, MA. Carbachol, atropine, isoproterenol, IBMX, ascorbate and LiCl were obtained from the Sigma Chemical Company, St. Louis, MO. All other chemicals were reagent grade. Eggs were obtained from McIntyre Poultry and Eggs, San Diego, CA.

RESULTS

To determine the ages at which the phosphoinositide response is coupled to muscarinic receptor activation, hearts from 4-, 6-, 9-, 12-, and 13-day embryos were incubated with [^{3}H]inositol as described in Methods. Samples were then treated with 30 μM carbachol.

Figure 1 shows that carbachol-stimulated phosphoinositide hydrolysis, as measured by the accumulation of [^{3}H]Ins1P, occurred at all ages examined. The [^{3}H]Ins1P accumulated per mg protein or per heart (not shown) was greatest at early ages and decreased with increasing age. The stimulation of

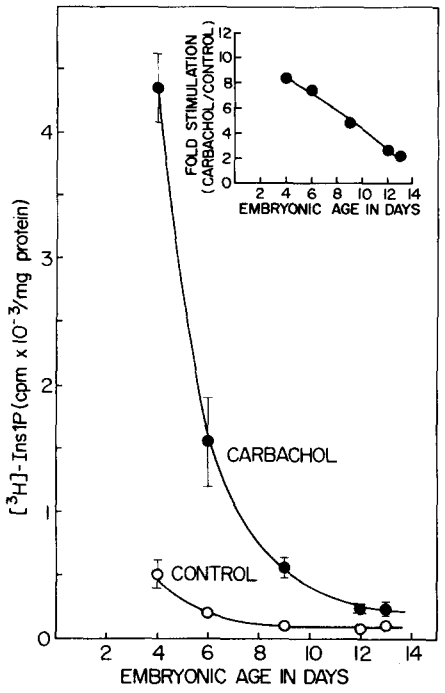

Fig. 1. Development of carbachol-stimulated phosphoinositide hydrolysis in embryonic chick heart. Results are means ± S.E., N = 5–6. Key: control, 10 mM LiCl (○); and 30 μM carbachol plus 10 mM LiCl (●). Inset: Fold stimulation of phosphoinositide hydrolysis.

phosphoinositide breakdown by carbachol was 8-fold at day 4 and decreased to 2-fold by day 13. The effect of 30 μM carbachol on 4-day-old hearts was blocked completely by 10 μM atropine, a muscarinic antagonist (data not shown).

The concentration of carbachol used in the experiments shown in Fig. 1 is not maximal for stimulating [^{3}H]Ins1P formation in 13-day hearts [11]. To determine if the apparently increased hydrolysis of phosphoinositide at 4 days reflects a change in maximal capacity or in sensitivity to muscarinic agonists, a concentration–response curve for agonist stimulation of phosphoinositide hydrolysis was generated for 4-day-old embryonic hearts. The results of this experiment are shown in Fig. 2. Half-maximal stimulation by carbachol occurred at 30 μM, and maximal stimulation occurred at 1 mM. These are identical to the concentrations necessary for stimulating [^{3}H]-Ins1P formation in 13-day hearts [11]. Therefore, while the amount of [^{3}H]Ins1P generated in response to carbachol was larger in 4-day hearts than in older hearts, the sensitivity to agonists at the two stages was identical.

The other biochemical response studied was muscarinic inhibition of catecholamine-stimulated cyclic AMP formation. To determine the course of development of this response, 4-, 6-, 9-, 12- and 13-day embryonic hearts were incubated as described in Methods. Figure 3 shows that the β-adrenergic agonist isoproterenol (3 μM) stimulated the formation of cyclic AMP in hearts of all ages studied. The amount of cyclic AMP generated with iso-

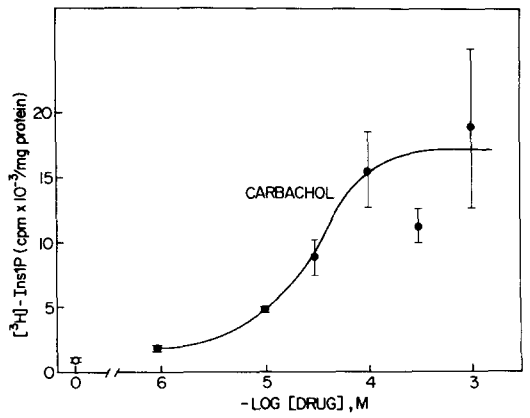

Fig. 2. Dose effects of carbachol on phosphoinositide hydrolysis in 4-day hearts. Results are means ± S.E., N = 4–6. Key: control, 10 mM LiCl (○); and carbachol plus 10 mM LiCl (●).

proterenol alone was larger at young ages than at older ages. Basal cyclic AMP levels did not change with age. Carbachol (30 μM) had no effect on basal cyclic AMP levels, but inhibited isoproterenol-stimulated formation of cyclic AMP at all ages. When the effect of carbachol was expressed as percent inhibition of isoproterenol-stimulated cyclic AMP formation, a value of approximately 75% was obtained at all ages.

The concentration–response relationship for muscarinic inhibition of isoproterenol-stimulated cyclic AMP formation in 4-day embryonic hearts was compared to the previously published results in 13-day hearts [11]. As shown in Fig. 4, carbachol inhibited isoproterenol-stimulated (3 μM) cyclic AMP formation in a concentration-dependent manner. Although carbachol alone had no effect on basal cyclic AMP levels, it inhibited isoproterenol-stimulated cyclic AMP formation half-maximally at 0.3 μM, and maximal inhibition was achieved with 30 μM carbachol. Studies on 13-day embryonic chick hearts yield identical values [11].

Our data on the effects of isoproterenol agree with previous reports [16–19] demonstrating that isoproterenol causes larger increases in cyclic AMP formation at early ages than at later ones. The data presented here indicate that carbachol is equally effective at inhibiting these increases at all ages studied.

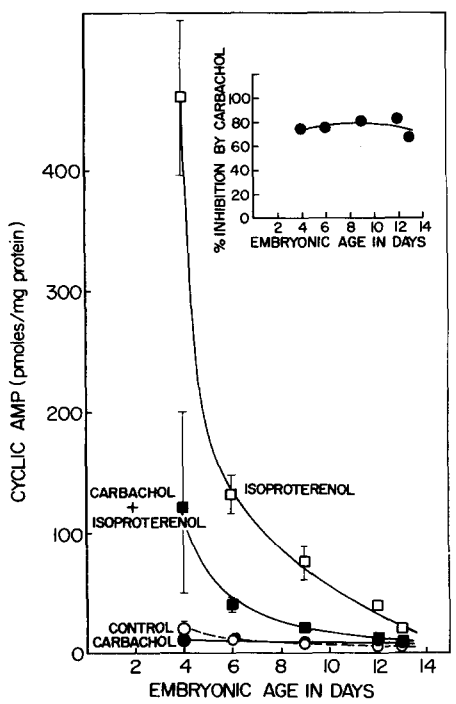

Fig. 3. Development of muscarinic inhibition of cyclic AMP formation. Results are means ± S.E., N = 3–4. Key: control (○); μM carbachol (●); 3 μM isoproterenol (□); 30 μM carbachol plus 3 μM isoproterenol (■). Inset: Percent inhibition by carbachol of isoproterenol-stimulated cyclic AMP formation (see Methods).

DISCUSSION

Muscarinic receptor stimulation caused both phosphoinositide hydrolysis and inhibition of isoproterenol-stimulated cyclic AMP formation in embryonic chick heart as early as 4 days *in ovo*. The sensitivities of the phosphoinositide responses to muscarinic agonists at 4 and 13 days were identical as were the sensitivities of the cyclic AMP responses. At both ages, there was a 100-fold greater potency of carbachol for inhibiting cyclic AMP formation than for stimulating phosphoinositide hydrolysis, a finding discussed in detail in a previous report [11].

The observation that the effect of carbachol on [^{3}H]InsIP formation was greater in young chick hearts than in older hearts suggests that phosphoinositides in young hearts are more readily available for, or more susceptible to, receptor-stimulated hydrolysis. A relationship between phosphoinositide hydrolysis and regulation of growth and differentiation has been suggested by a number of studies. For example, decreases in phosphoinositide hydrolysis occur upon contact inhibition of growth in fibroblasts [20] and upon differentiation of embryonic chick lens epithelial cells to lens fiber cells [21]. Conversely, increased phosphoinositide turnover is associated with growth stimulation in lymphocytes [22, 23] and fibroblasts [24]. The recent report of Sugimoto *et al.* [25] describing the phosphorylation of phosphatidylinositol to polyphosphoinositides by the Rous sarcoma transforming protein pp60^{src} gives additional weight to the hypothesis that phosphoinositides are somehow involved in growth regulation.

The age-dependence of the phosphoinositide response could also result from differences in specific labeling of a hormone-sensitive phosphoinositide pool with [^{3}H]inositol. The "pool" could be a particular cell type or an intracellular compartment. If phosphoinositides are labeled to a higher specific activity in 4-day hearts, the greater amount of [^{3}H]-InsIP formed would not necessarily reflect a greater concentration of metabolized phosphoinositide. In any event, our data indicate that the ability of the muscarinic receptor to stimulate phosphoinositide hydrolysis could not be the limiting factor in development of the physiological response to muscarinic agonists in the 4-day chick heart.

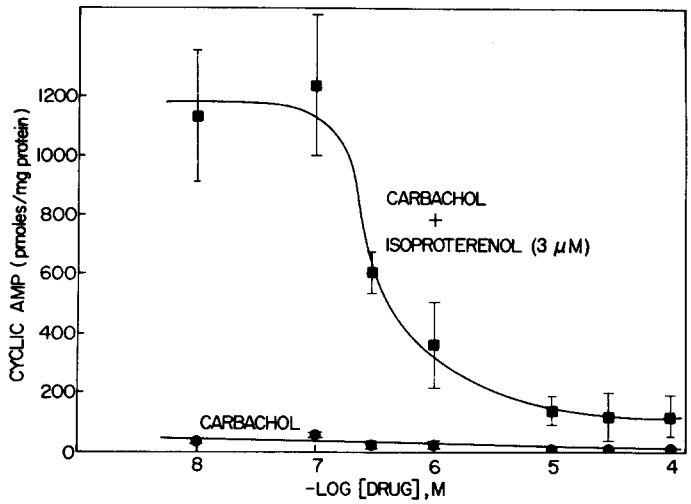

Fig. 4. Dose effects of carbachol on isoproterenol-stimulated cyclic AMP formation at 4 days. Results are means ± S.E., N = 3–6. Key: carbachol (●); and carbachol plus 3 μM isoproterenol (■).

The effect of age on catecholamine-stimulated cyclic AMP formation has been described by other investigators [16–19]. Our findings demonstrate the presence at early ages of an inhibitory muscarinic effect on cyclic AMP formation. This is in agreement with the finding of Nathanson that, in embryonic chick heart membranes, muscarinic agonists inhibit adenylate cyclase activity to the same extent and with virtually identical concentration-dependence throughout development [26]. These data indicate that muscarinic receptors can couple to adenylate cyclase and, therefore, that the inhibitory coupling protein N_i is likely to be present. Since there was also a constant 75% inhibition of isoproterenol-stimulated cyclic AMP formation at all ages, the development of this muscarinic receptor mechanism does not appear to explain ontogenetic changes in the functional responses to acetylcholine.

In summary, both phosphoinositide hydrolysis and inhibition of catecholamine-stimulated cyclic AMP formation occurred in response to muscarinic agonists and have identical sensitivities at 4 and 13 days *in ovo*. Therefore, the lack of physiological responsiveness at early ages cannot be accounted for by the absence of one of these responses. A functional lesion may exist at a more distal step in either pathway, such as the absence of a required enzyme activity or defect in protein phosphorylation. Alternatively, effectors other than those studied here may signal the physiological change and be retarded in their development. Additional research will be required to differentiate between these possibilities.

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