Enhanced Coupling of Neonatal Muscarinic Receptors in Rat Brain to Phosphoinositide Turnover

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Abstract: The relationship between the density of the muscarinic receptor in developing rat cerebral cortex and its coupling to phosphoinositide turnover is examined. Tissue slices from rats of various ages were incubated with myo-[2-^{3}H]inositol, and the effect of carbamoylcholine on the release of total inositol phosphates was determined. Binding of [^{3}H]quinuclidinyl benzilate was determined in the same tissue. Although muscarinic receptor density in day-18 embryonic cortex was only 5% of that in the adult, the maximal response of stimulated phosphoinositide turnover to carbamoylcholine (1–10 mM) was at the adult level (i.e., three-fold increase). Comparison of the dependence of the turnover on carbamoylcholine concentration revealed that in neonates, the dose–response curve was shifted to the left, giving a half-maximal effect at concentrations approximately tenfold lower than that in the adult. In addition, the partial muscarinic agonists oxotremorine-2 and bethanechol were both more efficacious in young rats than in adults. The differences could not be accounted for either by alterations in agonist affinity for the receptor or by the presence of "spare" muscarinic receptors. These results indicate that muscarinic receptors in fetal and newborn rat cerebral cortex are more efficiently coupled to stimulation of phosphoinositide turnover than in the adult. **Key Words:** Inositol phospholipid—Muscarinic receptors—Rat cerebral cortex development. **Heacock A. M. et al.** Enhanced coupling of neonatal muscarinic receptors in rat brain to phosphoinositide turnover. *J. Neurochem.* **48**, 1904–1911 (1987).

At birth, rat brain muscarinic acetylcholine receptor (mAChR) density is very low (10% of adults), as is the activity of the presynaptic marker choline acetyltransferase (Coyle and Yamamura, 1976; Kuhar et al., 1980). Activation of the mAChR in adult brain (Hokin and Hokin, 1955) results in the stimulation of the turnover of inositol phospholipids and leads to the formation of the second messengers inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (Berridge and Irvine, 1984; Nishizuka, 1984). Little is known about the functional coupling of the mAChR at early stages in rat brain development, although muscarinic stimulation of phosphoinositide turnover has been observed in 7-day-old cerebral cortex (Reddy and Sastry, 1979; Lee et al., 1985) and in 6-day-old hippocampus (Nicoletti et al., 1986). The developing CNS thus offers a useful model system in which to examine the relationship between mAChR density and the magnitude of stimulated phosphoinositide turnover. This study was undertaken to determine at which stage of development mAChRs in fetal and newborn rat cere-

bral cortex are coupled to phosphoinositide turnover and to explore the possibility of differences in coupling characteristics during development. The results indicate that mAChRs of 18-day-old embryonic cerebral cortex, the earliest examined, are linked to stimulated inositol lipid hydrolysis. Moreover, although few receptors are present in fetal and neonatal rat brain, they appear to be more efficiently coupled to the phosphoinositide response than are adult mAChRs. A preliminary report of this study has appeared in abstract form (Heacock et al., 1986).

MATERIALS AND METHODS

Carbamoylcholine (carbachol), bethanechol, atropine, and arterenol (noradrenaline) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); ibotenic acid was obtained from Regis Chemical Co. (Morton Grove, IL, U.S.A.); myo-[2-^{3}H]inositol (15 Ci/mmol) was purchased from ARC (St. Louis, MO, U.S.A.); and L-[^{3}H]quinuclidinyl benzilate (QNB; 33.1 Ci/mmol) and propylbenzylcholine mustard (PrBCM) were purchased from New England

Nuclear (Boston, MA, U.S.A.). Dowex-1 × 8 (100–200 mesh; in the formate form) was obtained from BioRad (Rockville Centre, NY, U.S.A.).

Release of labeled inositol phosphates from brain slices

Wistar rats of the indicated ages were killed by decapitation, the cerebral cortex was dissected, and cross-chopped slices (350 × 350 μm) prepared on a McIlwain tissue chopper. For the newborn rats, tissue pooled from eight to 12 animals was used for each experiment. Adult (6–10 weeks, male) samples included tissue from one to two animals. A continuous labeling paradigm (Berridge et al., 1982) was carried out essentially as previously described (Fisher and Bartus, 1985). This was chosen, rather than prelabeling, because of its greater sensitivity and decreased handling of the fragile neonatal tissue. Tissue slices were washed five times for a total of 30–45 min in 10–20 ml of warm buffer A [142 mM NaCl; 5.6 mM KCl; 2.2 mM CaCl$_2$; 3.6 mM NaHCO$_3$; 1 mM MgCl$_2$; 5.6 mM glucose; and 30 mM N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (HEPES buffer, pH 7.4)]. Fifty microliter aliquots of tissue slices (0.6–1.1 mg of protein) were incubated for 120 min at 37°C in buffer A, which contained 3 μCi (0.4 μM) myo-[2-^{3}H]inositol, 15 mM LiCl, and the indicated agonists (or buffer A for controls), in a total volume of 0.5 ml (Berridge et al., 1982). Reactions were terminated by the addition of 1.5 ml of chloroform/methanol (1:2, by vol). The water-soluble total inositol phosphates (IPs) were separated from myo-[2-^{3}H]inositol by ion-exchange chromatography and quantitated as previously described (Fisher et al., 1984). An aliquot of the lower organic phase was removed to determine the incorporation of myo-[2-^{3}H]inositol into phospholipid. Data are expressed as dpm [^{3}H]IPs per 10^4 dpm [^{3}H]inositol lipids. In some experiments, radioactivity in individual IP species was determined following sequential elution of Dowex-1 (formate form) ion-exchange columns (Berridge et al., 1983). For both neonatal and adult rats, most of the radioactivity was in inositol 1-phosphate (IP$_1$), as previously noted for guinea pig brain (Fisher and Bartus, 1985), with only 0.3–0.6% of the radioactivity in IP$_3$. Prior to each experiment, the myo-[2-^{3}H]inositol substrate was passed through a similar column to remove possible polar contaminants. Data for stimulated IP release as a function of agonist concentration were analyzed as described previously by means of a computer curve-fitting program (Fisher et al., 1983) that gives the concentration of agonist required for one-half maximal stimulation of IP release (EC$_{50}$). Data are presented as mean ± SEM. Statistical significance was evaluated by means of the Student's t test.

[^{3}H]QNB binding to brain homogenates

Homogenates of freshly dissected cerebral cortex were prepared in 10 mM sodium phosphate buffer (pH 7.4) and 100–400 μg of protein incubated in 2 ml of buffer A at 20°C with 1.0 nM L-[^{3}H]QNB. Reactions were terminated after 90 min by vacuum filtration through GF/B filters, and radioactivity was quantitated as previously described (Fisher et al., 1983). Specific binding was calculated by subtraction of radioactivity bound in the presence of 10 μM atropine. Analysis of [^{3}H]QNB binding to tissue homogenates and its competition by carbachol was performed by a computer curve-fitting program that generates values for concentrations of high- and low-affinity forms of mAChR, with the corresponding affinities K_H and K_L, and the percentages of

high- and low-affinity sites (McKinney and Coyle, 1982; Fisher et al., 1983). The data were fitted to both a one- and two-site model (i.e., one or two affinity forms of the receptor) and best fit determined by a minimum sum of the squared residuals and a Student's t test on the differences (significance at p < 0.05). The data were also examined for a systematic deviation from a one- or two-site model by nearest neighbor residual analysis (Birdsall et al., 1978).

Protein was measured by the method of Geiger and Bessman (1972).

Receptor alkylation

PrBCM, at a concentration of 0.2 mM, was cyclized in 10 mM sodium phosphate buffer (pH 7.4) for 45 min at room temperature to form the aziridium ion (Young et al., 1972). Brain slices suspended in buffer A were then incubated with PrBCM (2 × 10^{-9} to 2 × 10^{-8} M) at 37°C for 15 min. Following extensive washing of the slices with buffer A at 37°C to remove unbound PrBCM, aliquots were either homogenized for determination of QNB binding or incubated with myo-[2-^{3}H]inositol, as described above, in the presence or absence of 1 mM carbachol. Although some recovery from mAChR inactivation is reported to occur (Young et al., 1972), preliminary studies have established that the degree of receptor occlusion remains constant during the measurement of phosphoinositide turnover (S. K. Fisher, unpublished observations).

RESULTS

Comparison of mAChR density and stimulated IP release

Incorporation of myo-[2-^{3}H]inositol into inositol lipids and basal release of [^{3}H]IPs increased in a linear or nearly linear manner during the 2-h incubation period (data not shown). Initial experiments revealed age-related differences in inositol lipid labeling and a concomitant decline with age of basal release of [^{3}H]IPs from 3494 ± 174 dpm/mg protein in the neonate to 1,668 ± 123 dpm/mg protein in the adult (n = 4, p < 0.05; see also Nicoletti et al., 1986). Since basal release of [^{3}H]IPs is proportional to the amount of [^{3}H]inositol incorporated into lipids, this difference was no longer apparent if the basal values for unstimulated IP release were normalized to lipid labeling: 1-day-old cortex = 421 ± 65 and adult cortex = 439 ± 112 dpm [^{3}H]IP/10^4 dpm ^{3}H-lipid (see also Table 1). Thus, the latter method of calculation offers a more valid means of comparison and was used for all of the data presented here. Cortical tissues from 18-day-old embryos, newborn, and adult rats were labeled with myo-[2-^{3}H]inositol, and the ability of carbachol to enhance release of [^{3}H]IPs was assessed (Fig. 1A). Tissue from the same animals was also assayed for [^{3}H]QNB binding (Fig. 1B). The concentration of carbachol necessary to achieve maximal stimulation of IP release varied among the groups of animals from 1 to 10 mM. This stimulation was blocked (75–100%) by inclusion of atropine (10^{-5} M; not shown). Examination of the individual IP species revealed the predominant species in stimulated tissue, as under basal conditions, was IP$_1$, whereas only small increases (40–

TABLE 1. *Ontogeny of agonist-stimulated phosphoinositide turnover*

	Radioactivity in IPs (dpm/10⁴ dpm ³H-lipid)			
Age	Control	CCh	IBO	NA
1 day	244 ± 36	1,368 ± 35	2,398 ± 158	722 ± 151
7 day	259 ± 11	2,834 ± 588	1,742 ± 333	584 ± 12
11 day	257 ± 34	2,737 ± 33	1,879 ± 249	981 ± 21
Adult	270 ± 15	1,039 ± 124	617 ± 15	1,451 ± 81

Slices of cerebral cortex from rats of the indicated ages were incubated with [³H]inositol in the absence (control) or presence of one of the following agonists: carbachol (CCh; 10 mM), ibotenic acid (IBO; 1 mM), or norepinephrine (NA; 0.1 mM). After 2 h, reactions were terminated by the addition of 1.5 ml chloroform-methanol (1:2, by vol), and radioactivity in the IPs fraction and in inositol lipids was determined as described in Materials and Methods. Basal labeling of ³H-lipid (dpm/mg protein) in 1-, 7-, 11-day-old and adult tissue was 5.7 × 10⁴, 4.0 × 10⁴, 3.7 × 10⁴, and 2.7 × 10⁴, respectively. Only minimal alterations in lipid labeling were observed on addition of agonist. Values shown are means (±SEM) of triplicate samples from one of two similar experiments.

50%) in [³H]IP₃ release in response to carbachol were observed.

Although mAChR density showed a progressive increase during development, reaching adult levels by 6
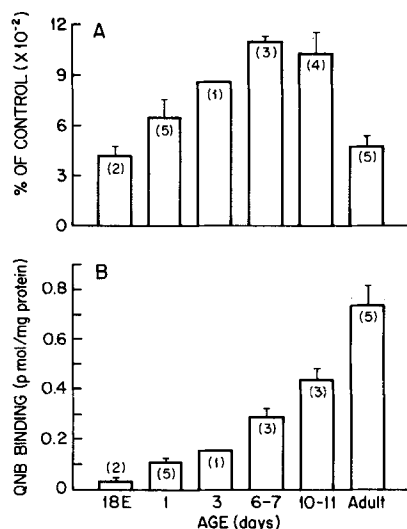

FIG. 1. Developmental changes in carbachol-stimulated [³H]IP release (**A**) and mAChR concentration (**B**) in rat cerebral cortex. A: Tissue slices from 18-day-old embryos (18E), newborn (1–11 days postnatal), and adult rats were incubated in triplicate with 3 μCi [³H]inositol and 15 mM LiCl in the presence of 0–10 mM carbachol. Reactions were terminated after 2 h, and radioactivity in the IP fraction and in inositol lipids was determined. Data are calculated as dpm [³H]IP/10⁴ dpm ³H-lipid and are expressed as percent of control (×10⁻²). Values at 6–11 days were significantly different from adult (p < 0.01), whereas values at 1 day were not. B: Binding of [³H]QNB to tissue homogenates (100–400 μg protein) was determined by a filtration assay following incubation with 1 nM [³H]QNB for 90 min in the presence or absence of 10 μM atropine. Values shown are mean ± SEM (or range, for n = 2) for the number of experiments indicated.
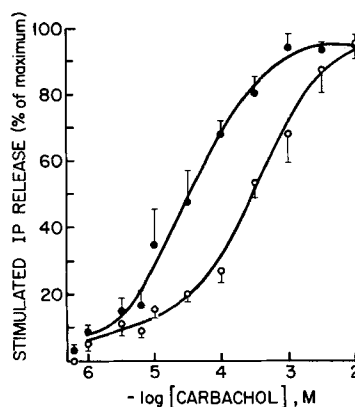

FIG. 2. Dose–response curves for stimulated release of IPs in 1-day-old (●) and adult (○) rat cortex. Tissue slices were incubated with 3 μCi myo-[2-³H]inositol in the presence of the indicated concentrations of carbachol. Reactions were terminated after 120 min, and radioactivity in the IPs fraction was determined. Data are expressed as a percentage of the maximum response (shown in Fig. 1). Each value is the mean ± SEM of four individual experiments. Analysis of the data by a computer curve-fitting program showed a one-site interaction at both ages, although the Hill coefficients were somewhat less than 1, 0.66, and 0.68 for 1-day-old and adult tissue, respectively.

weeks of age, an "adult" [³H]IP response to carbachol (three- to fourfold increase) was present in the youngest animals examined, 18-day-old embryos, a time when [³H]QNB binding was <5% of adult values. The magnitude of the carbachol response continued to increase, reaching twice that of the adult response at 6–11 days postnatal. Addition of carbachol led to small increases in inositol lipid labeling in 1-day-old cortex but not in adult cortex, 125 ± 4% and 100 ± 4% of control, respectively (mean ± SEM for five to eight separate experiments). Thus, if [³H]IP release had been normalized to protein content, carbachol-stimulated release in the newborn would have appeared to be even greater than the fourfold increase (Fig. 1) calculated by normalizing to lipid labeling.

Dose–response for carbachol and effect of partial muscarinic agonists

Comparison of the carbachol concentration curves for 1-day-old rat cortex and adult rat (6–10 weeks) cortex (Fig. 2) revealed that in the newborn animals, the dose–response curve was shifted to the left, with an EC₅₀ (mean ± SEM) of 39 ± 8 μM, compared with the adult value of 345 ± 115 μM (p < 0.05). Comparably low EC₅₀ values were also found in 18-day-old embryonic and 3-day-old postnatal cortex (Fig. 3A), whereas the shift toward adult values appeared to begin at 6 days and be completed by 10 days postnatal. The changes in EC₅₀ were accompanied by alterations in the responses to the partial muscarinic agonists oxotremorine-2 and bethanechol (Fig. 3B and C), both of which were more efficacious in the young rats than in the adults.
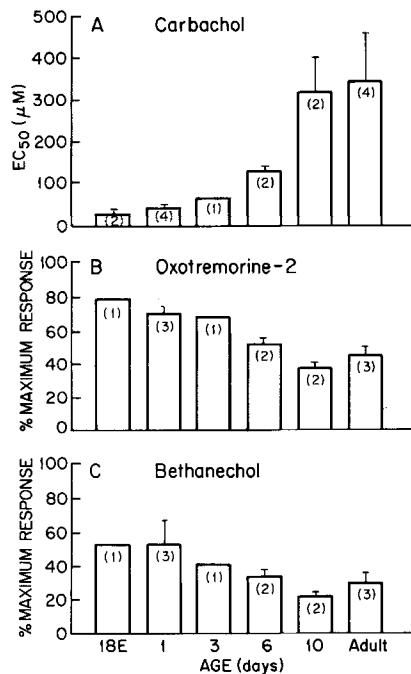
FIG. 3. Developmental changes in the EC_{50} for stimulation of IP release by carbachol **(A)** and the efficacy of the partial agonists oxotremorine-2 **(B)** and bethanechol **(C)**. A: Determination of the EC_{50} for carbachol was carried out as described in Fig. 2 for cerebral cortical tissue from rats of the indicated ages. The mean EC_{50} for 1-day-old cortex was significantly different from adult cortex (p < 0.05). The EC_{50} was calculated by a computer curve-fitting program. B and C: Tissue slices from rats of the indicated ages were incubated with myo-[2-^{3}H]inositol in the presence of optimal concentrations of the partial muscarinic agonists oxotremorine-2 (1 mM) or bethanechol (30 mM). Reactions were terminated after 2 h, and radioactivity in inositol phosphates was determined. Results are expressed as a percentage of the maximum response obtained with carbachol (1–10 mM). Values shown are means ± SEM (or range, for n = 2) of the indicated number of experiments. Differences between 1-day-old and adult cortex were not statistically significant for bethanechol but were for oxotremorine-2 (p < 0.002). (For structure of oxotremorine-2, see Fisher et al., 1984.)

Carbachol displacement of [^{3}H]QNB binding

The possibility that the low EC_{50} for stimulated IP release by carbachol in newborns is accounted for by a higher affinity of the newborn mAChR for binding of carbachol was assessed by determining the ability of carbachol to displace [^{3}H]QNB from homogenates of 1-day-old or adult rat cortex (Fig. 4). The percentage of high-affinity sites and the K_L values for the two groups did not differ; however, the K_H for 1-day-old cortex appeared to be greater than that for the adult animals (p < 0.05). Thus, the differences in carbachol EC_{50} for stimulated phosphoinositide turnover seem not to reflect altered agonist affinities for the mAChR.

Effect of alkylation of neonatal mAChRs

The apparent shift in EC_{50}, as well as the efficacy of partial agonists in neonatal cortex, could be interpreted to indicate the presence of "spare" receptors

for the phosphoinositide response. This possibility was explored by partial alkylation of mAChRs in 1-day-old rat cortex with PrBCM and subsequent comparison of the residual mAChR density with the ability of carbachol to stimulate [^{3}H]IP release (Fig. 5). [^{3}H]QNB binding declined in parallel with the IP response ($r = 0.91$). Intersection with the ordinate above the origin might indicate that some of the mAChRs in the slice preparation are not accessible to carbachol during phosphoinositide turnover measurement, as has been suggested for α_1-adrenergic receptors during the norepinephrine-stimulated inositol lipid hydrolysis in rat cerebral cortex slices (Johnson and Minneman, 1985).

Comparison with other agonists

To assess whether the magnitude of carbachol-stimulated IP release in neonatal tissue might be due to a general hypersensitivity of phosphoinositide turnover to receptor activation, the effects of norepinephrine and the glutamate analog, ibotenic acid, were determined and compared with those of carbachol (Table 1). Each displayed a unique developmental profile. In contrast to the equivalent stimulation of IP release in newborns and adults by carbachol, the efficacy of ibotenate showed a substantial decline with age, whereas that of norepinephrine increased.

DISCUSSION

Evidence is accumulating in support of a major role for inositol lipid breakdown in receptor-mediated transmembrane signaling in the CNS (Berridge and Irvine, 1984; Nishizuka, 1984). mAChR-mediated stimulation of phosphoinositide turnover has been characterized both biochemically and pharmacologically (Fisher and Agranoff, 1985; Fisher, 1986). The mAChR is proposed to exist in at least two interconvertible forms of high and low affinities (Birdsall et al., 1978). In the CNS, mAChRs appear to be tightly coupled to phosphoinositide turnover in that there is a close relationship between receptor occupancy and stimulated inositol lipid hydrolysis (Fisher, 1986).

In fetal and neonatal rat brain, low levels of mAChR are present before cholinergic innervation occurs (Coyle and Yamamura, 1976; Kuhar et al., 1980), a time when one might predict little need for the receptors to be functionally coupled to a transmembrane signaling pathway. Contrary to this expectation, activation of the few receptors present in developing rat cerebral cortex elicited as large an increase in phosphoinositide turnover as is observed in the adult (Fig. 1). Calculated as ratios of carbachol-stimulated IP release to mAChR density, the responses of 18-day-old embryonic and 1-day-old postnatal rats are, respectively, 20- and 10-fold greater than that in adult brain. Studies of mAChR function in embryonic chick heart (Orellana and Brown, 1985) and retina (Large et al., 1985a) have similarly revealed coupling of the receptors to phosphoinositide turnover prior to

FIG. 4. Occupancy of mAChR in 1-day-old (**A**) and adult (**B**) rat cerebral cortex by carbachol. Aliquots of homogenates of cerebral cortex (100–400 μg protein) were incubated with L-(−)[³H]QNB (1.0 n*M* in A; 1.13 n*M* in B) in the presence of various concentrations of carbachol. The displacement of [³H]QNB binding was analyzed for a one- or two-site interaction by a computer program that generated plots of theoretical one-site (O) and two-site (●) fits. These are compared to the actual (△) receptor occupancy data. Similar affinity constants for [³H]QNB (K_Q), 78 and 86 p*M*, were obtained for 1-day-old and adult cortex. Carbachol binding to mAChR in both tissues fit the criteria for a two-site model (see Materials and Methods). Results shown are from one of four separate experiments that gave similar results. Mean values for K_H, K_L, and percent high-affinity sites (±SEM) were 4.2 ± 1.3 μ*M*, 212 ± 19 μ*M*, and 28 ± 4% for 1-day-old cortex, and 0.8 ± 3 μ*M*, 250 ± 51 μ*M*, and 24 ± 3% for adult cortex; K_H for 1-day-old cortex was significantly different from that of the adult cortex (p < 0.05).
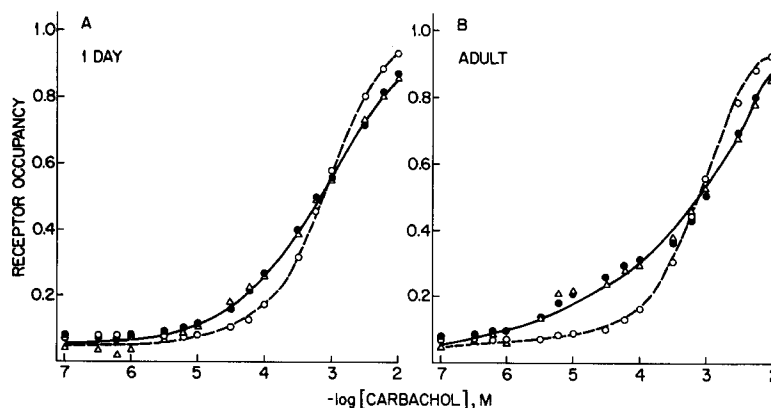
innervation. In avian heart, the ability of carbachol to stimulate IP release appears to decline with age (Orellana and Brown, 1985).

A further difference in the coupling characteristics of neonatal and adult mAChRs was revealed by examination of the carbachol dose–response curves (Fig. 2). The concentration of carbachol required to elicit a half-maximal phosphoinositide response was approximately tenfold lower in newborns than in adults. This observation might be accounted for by alterations in the binding of the agonist to the two affinity forms of the mAChR. Although there is a report that the high-affinity site is absent from 1-day-old rat cortex (Kuhar

FIG. 5. Correlation of mAChR receptor density with carbachol-stimulated IP release. Slices of cerebral cortex from 1-day-old rats were alkylated with various concentrations of PrBCM (2 × 10⁻⁹ to 2 × 10⁻⁸ *M*), as described in Materials and Methods. Following washing of the tissue slices, residual [³H]QNB binding and the ability of carbachol (1 m*M*) to stimulate IP release was assessed. There was a significant correlation (*r* = 0.91, p < 0.01) between receptor loss and loss of stimulated IP release. Results were obtained from three separate experiments.

et al., 1980), we found that 1-day-old and adult rat cortex contained a similar proportion of high-affinity sites. In addition, there was no enhancement of agonist binding affinity for either high- or low-affinity sites in 1-day-old tissue. Indeed, there appears to be a reduced carbachol affinity for the high-affinity sites in 1-day-old cortex (Fig. 4), a finding similar to that reported for the developing avian retina (Large et al., 1985*a*). It remains possible that affinity constants obtained from tissue homogenates do not accurately reflect agonist affinities of intact tissue slices, although comparisons of intact and homogenized N1E-115 neuroblastoma cells revealed no difference in muscarinic agonist binding parameters (McKinney et al., 1985). Precise comparisons between the agonist affinity constants and the carbachol EC₅₀ for phosphoinositide turnover are not possible, since the binding assays were carried out at 20°C in the absence of Li⁺, whereas the IP release assays were carried out at 37°C in the presence of Li⁺; however, inclusion of 15 m*M* LiCl in the QNB assay did not alter the binding characteristics of either newborn or adult cortical tissue (data not shown). Decreasing temperature reportedly results in small decreases in the binding constants for the mAChR in neuroblastoma N1E-115 cells (McKinney et al., 1985). Thus, differential temperature effects on neonatal and adult mAChR binding characteristics cannot be ruled out, although they would not likely be of sufficient magnitude to affect the conclusion that differences in the affinity of the mAChR for carbachol do not account for differences in the EC₅₀ for phosphoinositide turnover.

The absence of marked changes in the affinity of the mAChR for carbachol in tissue exhibiting an enhanced agonist efficacy for phosphoinositide turnover might indicate the presence of spare receptors, i.e., submaximal receptor occupancy required for maxi-
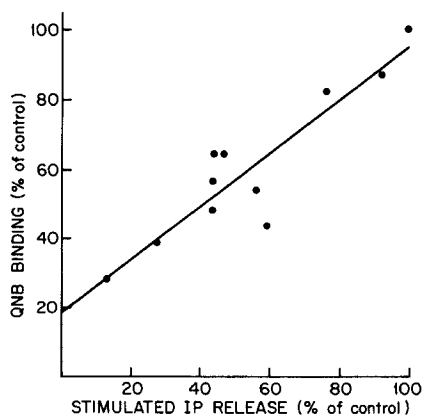
mal response. The greater efficacy of the partial muscarinic agonists oxotremorine-2 and bethanechol (Fig. 3) is also consistent with this possibility. As observed for adult brain (Fisher and Snider, 1986), irreversible inactivation of the mAChR by alkylation with PrBCM results in a parallel loss of receptor number and carbachol-stimulated IP release (Fig. 5). Thus, in neonatal as in adult brain, there is little evidence of mAChR reserve for inositol lipid hydrolysis.

The developmental profile of carbachol-stimulated phosphoinositide turnover in rat cerebral cortex contrasts markedly with that of two other phosphoinositide-linked agonists examined. In agreement with the observations of Schoepp and Rutledge (1985) and Nicoletti et al. (1986), we find that the response to norepinephrine increases during development, whereas that of the glutamate analog, ibotenic acid, decreases (Table 1). Nicoletti et al. (1986) also find a robust phosphoinositide response elicited by addition of carbachol to 6-day-old rat hippocampus, with no substantial change during development. Comparison with these other agonists may prove useful in elucidating the mechanism of the observed enhanced coupling of fetal and neonatal mAChRs. For example, there is indirect evidence indicating that guanine nucleotide-binding regulatory proteins (G-proteins) play a role in receptor coupling to phosphoinositide turnover (Gonzales and Crews, 1985; Joseph, 1985; Litosch et al., 1985). G-proteins have been found to interact with the mAChR (Florio and Sternweiss, 1985) and with α_1-adrenergic receptors (Goodhart et al., 1982) and guanine nucleotides alter the binding of agonists to the mAChR (Ehlert et al., 1981). It has been suggested that a lack of such a regulatory protein might account for the limited coupling of α_1-adrenergic receptors to inositol lipid turnover in 7- and 14-day-old rat cortex (Schoepp and Rutledge, 1985). Similarly, the progressive reduction in the ability of glutamate and ibotenic acid to stimulate IP release in developing rat hippocampus (Nicoletti et al., 1986) may reflect the appearance of a negative or inhibitory coupling factor. The results described here for the mAChR could indicate the presence of a positive coupler early in development, which subsequently declines. Alternatively, there may be a negative coupler that appears later in development and serves to diminish the signal produced by the increased numbers of mAChRs. Precedent for such a mechanism can be found in recent studies in which alterations in the coupling of heart α_1-adrenergic receptors to contraction, on the one hand (Steinberg et al., 1985), and the coupling of heart mAChRs to adenylate cyclase inhibition (Liang et al., 1986), on the other, could each be correlated with increased amounts of G-proteins. It may be that the greater sensitivity to muscarinic agonists in fetal and neonatal cortex constitutes a compensatory mechanism for the relatively low levels of acetylcholine encountered by the receptor at this stage

of development (Coyle and Yamamura, 1976). It should be noted, however, that the shift to adult EC_{50} values observed in the present study does not correspond closely to the developmental increase in cholinergic input to the cortex: At 10 days postnatal, the EC_{50} for carbachol stimulation of inositol lipid hydrolysis did not differ from the adult value, whereas cortical choline acetyltransferase activity is reportedly only 10% of that in the adult at this time (Coyle and Yamamura, 1976).

Relevant factors that might account for the enhanced coupling of the mAChR to phosphoinositide turnover, other than the postulated changes in regulatory proteins, include possible structural alterations in the mAChR or in its environment that might alter its coupling characteristics, e.g., changes in membrane fluidity (Rauch and Hitzemann, 1986) or in the anatomical localization of the receptor. Differences in the molecular weight and isoelectric point of the mAChR during development have been found in embryonic chick retina and rat olfactory bulb (Large et al., 1985a,b, 1986). Alternatively, the pool of inositol phospholipid coupled to the mAChR might be preferentially labeled with [^{3}H]inositol in neonatal brain, thus giving rise to an increased specific activity of released inositol phosphates. Data on the concentrations of the phosphoinositides in developing brain have recently been reviewed by Sun and Foudin (1985). In addition, it is likely that a common biochemical mechanism of mAChR-stimulated phosphoinositide turnover is shared by neonatal and adult cortex, i.e., phosphodiesteratic cleavage of phosphatidylinositol 4,5-bisphosphate with subsequent dephosphorylation of IP_3 and accumulation of IP_1 in the presence of Li^+ (Berridge, 1983; Berridge et al., 1983; Downes and Wusteman, 1983; Fisher et al., 1984); however, this mechanism has not been firmly established in brain (Hawthorne, 1986), and given the predominant release of IP_1 in both newborn and adult tissues, it remains possible that a secondary breakdown of phosphatidylinositol occurs in response to increased intracellular Ca^{2+} (Wilson et al., 1985).

Differences in both the affinity and efficacy with which muscarinic agonists enhance phosphoinositide turnover could well have broad significance in the understanding of higher brain function. Such differences have been reported previously in both neural and nonneural tissues (Brown and Brown, 1984; Ek and Nahorski, 1986; Evans et al., 1985; Fisher et al., 1983, 1984; Fisher and Bartus, 1985; Gil and Wolfe, 1985; Jacobson et al., 1985). The developing rat brain provides a longitudinal model that lends itself particularly well to tackling the problem of how mAChR coupling to second messengers is regulated.

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