Journal of Neuroscience Research 84:13–26 (2006)

Transmembrane Signaling Through Phospholipase C-β in the Developing Human Prefrontal Cortex

Iñigo Ruiz de Azúa,[1] Elena del Olmo,[2] Angel Pazos,[2] and Joan Sallés[1]*

[1]Department of Pharmacology, Faculty of Pharmacy, University of the Basque Country, Vitoria-Gasteiz, Spain
[2]Department of Physiology and Pharmacology, Faculty of Medicine, University of Cantabria, Santander, Spain

To investigate changes in muscarinic receptor-stimulated phospholipase C-β (PLC-β) activity during brain development, we examined the functional coupling of each of the three major protein components of the phosphoinositide system (M_1, M_3, and M_5 muscarinic receptor subtypes; $G_{q/11}$ proteins; PLC-β_{1-4} isoforms) in membrane preparations from post-mortem human prefrontal cerebral cortex collected at several stages of prenatal and postnatal development. In human prenatal brain membranes, PLC was found to be present and could be activated by calcium, but the ability of guanosine-5′-o-3 thiotriphosphate (GTPγS) or carbachol (in the presence of GTPγS) to modulate prenatal PLC-β was significantly weaker than that associated with postnatal PLC-β. Western blot analysis revealed that the levels of $G\alpha_{q/11}$ did not change significantly during development. In contrast, dramatically higher levels of expression of PLC-β_{1-4} isoforms and of M_1, M_3, and M_5 muscarinic receptors were detected in the child vs. the fetal brain, a finding that might underlie the observed increased activity of PLC. Thus, inositol phosphate production may be more efficiently regulated by altering the amount of effectors (PLC-β_{1-4}) and receptors ($M_{1,3,5}$ subtypes) than by altering the level of $G\alpha_{q/11}$ subunits. These results demonstrate that different PLC isoforms are expressed in the prefrontal cortex of the developing human brain in an age-specific manner, suggesting specific roles not only in synaptic transmission but also in the differentiation and maturation of neurons in the developing brain.

Key words: phospholipase C-β_{1-4} isoforms; $G\alpha_{q/11}$ sub units; muscarinic receptor subtypes; cerebral cortex; ontogenic development

The ontogeny of the cholinergic system in the human brain is of considerable interest in that it provides significant innervation to most regions of the adult brain and is closely related to motor and cognitive functions in humans (Perry, 1986). In addition, considerable evidence suggests that the cholinergic system plays a critical role in the regulation of cortical morphogenesis, as well as in cellular maturation and differentiation, in a number of mammalian species (for review see Hohmann and Berger-Sweeney, 1998). Various studies have described the developmental pattern of cholinergic markers, such as cholinergic enzymes and receptors in humans (Aguilar and Lunt, 1985; Egozi et al., 1986). However, whether different subtypes of muscarinic receptors have different developmental patterns is still unclear because of the lack of muscarinic receptor subtype-specific ligands.

One major pathway for neurotransmitter signaling involves phosphoinositide-specific, G-protein-dependent phospholipase C-β (PLC-β), which stimulates the formation of two second messenger system molecules, inositol triphosphate and diacylglycerol. Muscarinic receptor activation is well documented as one of the most robust activators of PLC-β in the brain (Crews et al., 1994). It is generally accepted that M_1, M_3, and M_5 muscarinic receptors couple preferentially via the pertussis toxin-insensitive $G_{q/11}$ protein to PLC-β. Several studies have examined the coupling of muscarinic receptors to PLC-β in the developing nervous systems of experimental animals (Rooney and Nahorski, 1989; Wall et al., 1992; Candura et al., 1995). However, details about this signal transduction pathway in the developing human brain are scarce and are limited by the experimental procedures employed (Larocca et al., 1994).

Experimental procedures used to measure the activity of the phosphoinositide system predominantly use intact cells with the substrate lipids prelabeled with [^{3}H]inositol prior to activation with appropriate agonists (Rooney and Nahorski, 1989; Wall et al., 1992; Larocca et al., 1994; Candura et al., 1995). In contrast, Claro and his colleagues optimized a method to measure phospho-

*Correspondence to: Dr. J. Sallés, Departamento de Farmacología, Facultad de Farmacia, Universidad del País Vasco/EHU, C/ Paseo de la Universidad No. 7, E-01006 Vitoria-Gasteiz, Spain.
E-mail: kfpsaalj@vc.ehu.es

Received 18 November 2005; Revised 1 March 2006; Accepted 1 March 2006

Published online 13 April 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.20858

TABLE I. Demographic Characteristics and Post-Mortem Delay of Subjects*

Human case	Age	Gender	Postmortem delay (hr)	Cause of death
W22	22 Weeks	Female	17	Prenatal distress
W35	35 Weeks	Male	N.D.[a]	Intrauterine death
W40	40 Weeks	Female	11	Prenatal distress
Y4	4 Years	Male	45	Motor-vehicle accident
Y8	8 Years	Male	24	Gunshot
Y13	13 Years	Male	19	Motor-vehicle accident
Y22	22 Years	Male	14	Motor-vehicle accident
Y23	23 Years	Male	32	Motor-vehicle accident
Y25	25 Years	Male	8	Motor-vehicle accident

*W22–35, 22–35 weeks of gestational age; Y4–13, 4–13, year old child; Y22–25, 22–25 year old adult.
[a]N.D., not determined. The subject died in the uterus.

inositide hydrolysis in rat brain cortical membranes by using exogenously labeled phosphoinositides, which exhibited relatively strong responses to several agonists (Claro et al., 1989; Wallace and Claro, 1990). With this method, each component of the phosphoinositide system can be measured: PLC-β can be activated directly with calcium, G-proteins can be activated with stable analogs of GTP [e.g., guanosine-5′-o-3 thiotriphosphate (GTPγS)] to stimulate PLC-β, and certain agonists in the presence of GTPγS stimulate the entire complex. By employing this method, post-mortem human brain membranes were shown to be responsive to stimulation by carbachol, dopamine, and serotonin (Wallace and Claro, 1993). Thus, examination of post-mortem membranes with exogenous substrates is currently one of the best available methods for examining the functionality of neurotransmitter systems that directly affect PLC-β in the human brain.

The present study was designed to measure the degree of activation of each component of the phosphoinositide complex in post-mortem human brain cortical membranes at several stages of prenatal and postnatal development. In addition, we also quantified the protein levels of several elements of the PLC-β transmembrane signaling complex by Western blot. In this way, we were able to examine the association between the levels of M_1, M_3, and M_5 muscarinic receptors; $G\alpha_{q/11}$ subunits; and PLC-β_{1-4} isoforms and their corresponding functional coupling in crude plasma membranes obtained from the same human brain sample.

MATERIALS AND METHODS

Subjects

Human brains were obtained at autopsy from three fetuses/newborns (prenatal cases), three children (postnatal cases), and three adults (adult cases; see Table I for demographic characteristics). These subjects did not show evidence of neurological or psychiatric disease nor of drug consumption on the basis of toxicological screening for psychotropic drugs and alcohol. The brains were removed at autopsy at the Pathology Service of the ''Marques de Valdecilla'' University Hospital. Tissue collection had been approved by the Ethical Committee of the hospital Forensic Institute for post-mortem human studies. The prefrontal cortex was promptly dissected and stored at –75°C.

Preparation of Human Brain Cortical Membranes

The preparation of membranes from human cortex samples was essentially as previously described by Garro et al. (2001). Prefrontal cortex samples stored at –75°C were thawed into a hypotonic buffer at 4°C containing 20 mM Tris-HCl, pH 7.0, with 1 mM EGTA (Tris/EGTA buffer) in the presence of protease inhibitors (1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 500 μM phenylmethylsulfonyl fluoride). The tissue was minced and homogenized in 10 volumes of Tris/EGTA buffer with a motor-driven Teflon pestle at its maximum setting (10 strokes at 1000 rev/min). Homogenates were centrifuged for 15 min at 39,000*g*, and pellets were washed three times and stored at –75°C until required for use. Protein was measured with the Bio-Rad dye reagent (Bio-Rad, Hercules, CA). When we measured muscarinic receptor subtype levels by immunoblotting, membrane samples were treated with N-glycosidase F (Boehringer Mannheim, Indianapolis, IN) prior to electrophoresis by using the method reported by Cali et al. (1994).

PLC Assay

PLC activity in brain cortical membranes was measured essentially as described previously (Claro et al., 1989; Garro et al., 2001, 2004) with 30 μM [^{3}H]phosphatidylinositol 4,5-bisphosphate ([^{3}H]PIP_2, Dupont New England Nuclear, Boston, MA) as substrate. The assay buffer contained 25 mM Tris-maleate, pH 6.8, 6 mM $MgCl_2$, 8 mM LiCl, 2 mM ATP, 1 mM sodium deoxycholate, 3 mM EGTA, and $CaCl_2$ to yield the desired final free Ca^{2+} concentration. The PLC assay volume was 100 μl, containing 100 μg of membrane protein. Reactions were initiated by addition of cold membranes to the prewarmed assay buffer containing the substrate and any other additions (GTPγS and carbachol). These assays were run for 15 min at 37°C, and were stopped by addition of 1.2 ml of chloroform:methanol (1:2, v/v), followed by 0.5 ml each of 0.25 mM HCI and chloroform, and the reaction mixtures were thoroughly vortexed. The phases were allowed to separate, and, after centrifugation, a 1-ml aliquot of the upper aqueous phase (containing the [^{3}H]inositol phos-

phates) was mixed with 4 ml of Optiphase HiSafe (EG&G, Inc.) for scintillation counting. Results referring to production of [^{3}H]inositol phosphates are expressed in picomoles and were derived from the specific radioactivity of the added [^{3}H]PIP_2.

Immunodetection of $G\alpha_{q/11}$ Subunits, PLC Isoforms, and Muscarinic Receptor Subtypes $M_{1,3,5}$

Western blot studies were performed as previously reported for PLC isoforms in human and rodent brain (Garro et al., 2001; Ruiz de Azúa et al., 2001; Sallés et al., 2001; López de Jesús et al., 2006). Frozen pellets of cortical membranes were resuspended in Tris/EGTA buffer in the presence of protease inhibitors, and aliquots (120 μl) of membrane suspension were added to 60 μl of 1 mM dithiothreitol (DTT) and 10% sodium dodecyl sulfate (SDS) and were boiled for 5 min. After incubation, 60 μl of 15 mM N-ethylmaleimide was added, and the samples were incubated for a further 15 min (21°C). Next, 60 μl of 25 mM DTT and 300 μl of sample buffer (25% glycerol, 5% β-mercaptoethanol, 2% SDS, 0.01% bromophenol blue, and 62.5 mM Tris-HC1, pH 6.8) were added and samples boiled for 5 min. Equal protein amounts were resolved by electrophoresis in 10% SDS-polyacrylamide gels. Proteins were transferred to polyvinylidine difluoride (PVDF) membranes (Amersham Pharmacia Spain). Equal loading of protein into each lane was verified by Ponceau staining of every blot. Blots were blocked in 5% nonfat dry milk/phosphate-buffered saline containing 0.5% bovine serum albumin (BSA) and 0.2% Tween (PBS-T) for 1 hr and were incubated overnight with PLC-β_1 antiserum (1:5,000 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and -β_2, -β_3, -β_4 antisera (1:1,000 dilution; Santa Cruz Biotechnology Inc.), PLC-γ antiserum (1:2,000 dilution; Transduction Laboratories, Lexington, KY), PLC-δ_1 antiserum (1:500 dilution; Transduction Laboratories), $\alpha_{q/11}$ antiserum (1:1,000 dilution; Santa Cruz Biotechnology Inc.), M_1 muscarinic receptor antiserum (1:500 dilution; Calbiochem), M_3 muscarinic receptor antiserum (1:500 dilution; Santa Cruz Biotechnology Inc.), or M_5 muscarinic receptor antiserum (1:500 dilution; Santa Cruz Biotechnology Inc.). Blots were washed and incubated with anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Amersham Pharmacia) diluted 1:4,000 in blocking buffer for 2 hr at room temperature. Immunoreactive bands were visualized with the enhanced chemiluminescence (ECL) system (Amersham Pharmacia). In all experiments, blots were subsequently incubated in stripping solution [62.5 mM Tris (pH 6.7), 2% (w/v) SDS and 100 mM β-mercaptoethanol] for 30 min at 50°C, washed twice with PBS-T, and reprobed with antiactin antibody (1:2,000 dilution; Sigma Chemical Co., St. Louis, MO) as described above, to test for nonspecific loading effects under our experimental conditions. The molecular mass of individual bands was measured by a prestained broad-range protein ladder (Novagen).

The amount of protein loaded for each protein was determined in pilot experiments to lie within the linear range of detection (see Garro et al., 2001; Ruiz de Azúa et al., 2001; Sallés et al., 2001; López de Jesús et al., 2006). Linearity regarding muscarinic receptors is shown in Figures 5–7. These aliquots (1–50 μg of membrane protein) were obtained from the same sample (adult human brain cortical membranes), subjected to electrophoresis on identical gels in duplicate, and immunoblotted at the same time under identical conditions. Identical samples from a control adult human brain (Y23) were run in all assays of fetal, child, and adult human brain, thereby allowing normalization of values as described in the figure legends.

Binding Assays

The specific binding of [^{3}H]3-quinuclidinyl benzilate (QNB; Dupont New England Nuclear) was measured as previously described in rat brain cortical membranes (Sallés et al., 1993a), with minor modifications. The assay buffer was phosphate-buffered saline (PBS; 40 mM Na_2HPO_4, 10 mM NaH_2PO_4, 100 mM NaCl, pH 7.4). For saturation experiments, appropriately diluted cortical membranes were added in a volume of 50 μl to test tubes with the appropriate concentration of [^{3}H]QNB (eight concentrations in the range of 12.5 pM to 5 nM). Routinely, the assay contained 62.5 μg of protein in a final volume of 2 ml PBS. Triplicate samples without and with 1 μM atropine served to establish total and nonspecific binding to receptors. Incubations were carried out for 45 min at 37°C. Binding reactions were terminated by rapid filtration over glass-fiber filters (Whatman GF/B) presoaked in 0.5% polyethylenimine and were rinsed rapidly with 4 ml of ice-cold 0.9% (w/v) NaCl solution. Radioactivity trapped on the filters was counted by liquid scintillation spectroscopy at an efficiency of 40%. For experiments involving the inhibition of [^{3}H]QNB specific binding by pirenzepine, a total concentration of approximately 0.3 nM of [^{3}H]QNB and 14–21 concentrations of pirenzepine (range 10 pM to 100 μM) were used.

Data Analysis

The data are usually presented as mean ± SE for the indicated number of experiments, carried out by using separate membrane preparations from each sample, performed either in triplicate (functional and binding assays) or in duplicate (Western blot assays). The significance of differences between means was analyzed by unpaired two-tailed Student's *t*-tests in the GraphPad Prism program (version 4.01 for Windows; GraphPad Software, San Diego, CA). Statistical significance was set at the 95% confidence level. The concentration-response curves for Ca^{2+}, GTPγS, and GTPγS plus carbachol were analyzed by nonlinear regression in GraphPad, which gave estimates of the basal level, maximal response (E_{max}), EC_{50}, and slope(n) of the curves.

Experiments in which the specific binding of a single concentration of [^{3}H]QNB were assayed in the presence of varying concentrations of pirenzepine were analyzed as competition curves by nonlinear regression analysis for models of one or two interacting sites using the appropriate equations in the GraphPad Prism program. The goodness of fit of the data to a two- vs. one-site receptor model was estimated by Snedecor's F-test. A model for two sites was retained only when it fit the data significantly better than a model for a single site ($P < 0.05$).

To evaluate the statistical significance of the differences between EC_{50} and K_D values, these parameters were logarithmically transformed ($pEC_{50} = -\log EC_{50}$ or $p\ K_D = -\log K_D$), because it has been previously demonstrated that parameters such as EC_{50} and affinity constants obtained experimentally are normally distributed after logarithmic transformation (Fleming et al., 1972).

RESULTS

Initial studies were conducted to examine the relative effects of GTPγS and carbachol on [^{3}H]PIP_2 hydrolysis in nine different preparations of human prefrontal cortical membranes. As previously reported for cortical membranes from the adult human brain (Garro et al., 2001), GTPγS (3 μM) significantly stimulated [^{3}H]PIP_2 hydrolysis, and this response was increased further with the addition of carbachol (1 mM). As shown in Figure 1A, the accumulation of [^{3}H]inositol phosphates induced by a 15-min incubation with 3 μM GTPγS was markedly greater in postnatal than in prenatal human brain membranes. Stimulation of PLC-β by carbachol (1 mM) in the presence of GTPγS is also shown in Figure 1A. Thus, carbachol was able to activate PLC-β in all the tissues examined, although the magnitude of the responses was markedly different, depending further of the stage of development.

The weak response of PLC-β to GTPγS and carbachol in fetal brain membranes could be due to a low level of phospholipase enzymatic activity at early stages of development. Alternatively, the enzymes could be active but uncoupled from the G-proteins and muscarinic receptors. In human and rodent brain cortical membranes, the sensitivity of PLC-β to GTPγS (in terms of nucleotide concentrations giving half-maximal stimulation) is significantly greater when assayed in the presence of a muscarinic receptor agonist (Claro et al., 1989; Sallés et al., 1993b; Garro et al., 2001; Ruiz de Azúa et al., 2001). Thus, we constructed complete concentration response curves for GTPγS in the absence and presence of carbachol (1 mM) for prenatal, postnatal, and adult membranes. Table II summarizes the data obtained, and Figure 1B shows representative prenatal, postnatal, and adult cases. Thus, in membranes prepared from fetal brain cases, there was a modest activation of PLC-β by GTPγS alone or in combination with carbachol, even at nucleotide concentrations as high as 100 μM (55% and 96% increases respectively, with respect to basal values). In contrast, in the case of child brain membrane preparations, maximal stimulation by GTPγS of 96% over basal was measured, and this was further increased by carbachol up to 180% (i.e., Fig. 1B, middle). Finally, in the case of adult brain membrane preparations, maximal stimulation by GTPγS of 137% over basal was measured, and this was further increased by carbachol to 166% (Fig. 1B, right). Moreover, the EC_{50} values for GTPγS were found to be significantly lower in child compared with fetal brain membranes (approximately 0.4 μM vs. 1.7 μM). Interestingly, carbachol induced only a twofold leftward shift in the apparent EC_{50} value for GTPγS in fetal brain membranes, whereas it induced a tenfold leftward shift in child brain membranes and a fourfold leftward shift in adult brain membranes (see Table II).

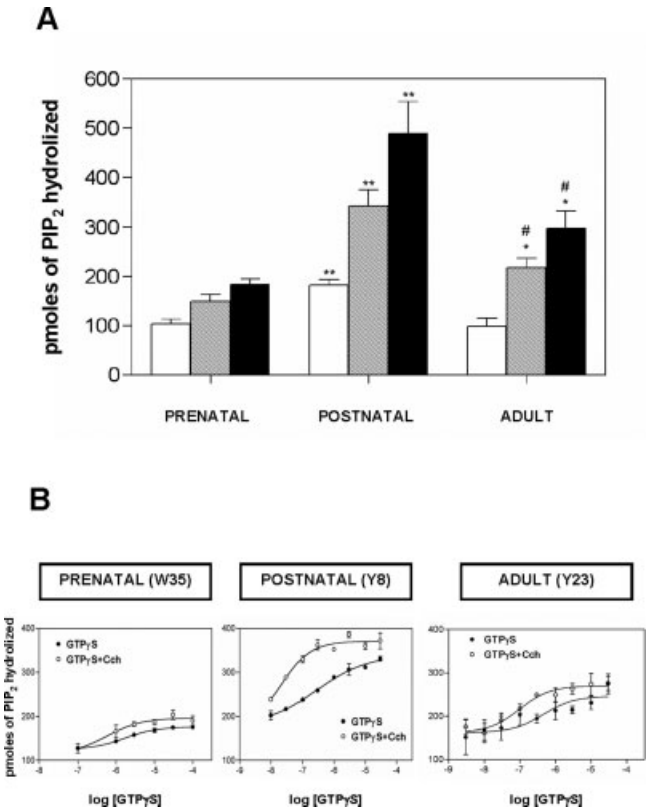

Fig. 1. Developmental profile of the stimulation of hydrolysis of exogenous [^{3}H]PIP_2 by carbachol and GTPγS in human cerebral cortical membranes. **A:** Human cerebral cortical membranes were incubated with exogenous substrate, in 50 nM free Ca^{2+} and no more additions for basal conditions (open bars) or in the presence of 3 μM GTPγS (shaded bars) or and 3 μM GTPγS plus 1 mM carbachol (solid bars). Results, expressed in picomols, are of three independent experiments carried out in triplicate. Each bar in the histogram represents the mean ± SEM of values for each individual belonging to the prenatal (W22, W35, and W40), postnatal (Y4, Y8, and Y13), or adult (Y22, Y23, and Y25) groups. $^*P < 0.05$, $^{**}P < 0.01$ prenatal vs. postnatal and adult; $^{\#}P < 0.05$ postnatal vs. adult (unpaired two-tailed Student's *t*-tests). **B:** Brain cortical membranes from a fetal (W35), a child (Y8), and an adult (Y23) case were incubated with different concentrations of GTPγS, in both the presence (open circles) and the absence (solid circles) of 1 mM carbachol. The free calcium concentration was 50 nM, buffered with 3 mM EGTA. Data in the left panel correspond to a representative experiment with prenatal case W35 (35 weeks of gestation); data in the middle panel are from a representative experiment with postnatal case Y8 (8 years old); data in the right panel correspond to a representative experiment with an adult case (Y23). PLC-β responsiveness to GTPγS and carbachol was found to be relatively weak in fetal membranes.

To determine whether the decreased functional responses in fetal cortical membranes were associated with specific changes in the density of PLC-β_{1-4} isoforms or $G\alpha_{q/11}$ subunits, we quantified these proteins in cortical

TABLE II. Data From the Concentration Effect Curves for the Effect of GTPγS on PLC-β Activity in Human Brain Cortical Membranes in the Absence and Presence of 1 mM Carbachol (Cch)[†]

	GTPγS alone		GTPγS + carbachol		
Human case	EC_{50} (μM)	E_{max} (pM)	EC_{50} (μM)	E_{max} (pM)	Δ
W22	1.45 ± 0.03	166.5 ± 8.2	0.72 ± 0.03	199.4 ± 2.3	2
W35	1.89 ± 0.04	151.4 ± 2.2	0.88 ± 0.04	190.2 ± 6.5	2
W40	1.88 ± 0.03	160.2 ± 3.3	0.92 ± 0.04	214.8 ± 8.7	2
Prenatal	1.74 ± 0.14***	159.4 ± 4.4***	0.84 ± 0.06***	201.5 ± 7.2***	2
Y4	0.41 ± 0.05	366.4 ± 7.8	0.043 ± 0.01	562.5 ± 7.8	10
Y8	0.38 ± 0.08	325.6 ± 3.2	0.038 ± 0.01	427.2 ± 11.4	10
Y13	0.43 ± 0.03	376.6 ± 2.2	0.045 ± 0.01	536.6 ± 10.4	10
Postnatal	0.41 ± 0.01	356.2 ± 15.6	0.04 ± 0.002	508.8 ± 41.5	10
Y22	0.43 ± 0.02	233.0 ± 7.5	0.093 ± 0.01	266.3 ± 2.4	4
Y23	0.38 ± 0.07	245.9 ± 12.6	0.091 ± 0.03	270.4 ± 4.4	4
Y25	0.41 ± 0.03	227.1 ± 16.4	0.097 ± 0.02	256.4 ± 3.0	4
Adult	0.41 ± 0.01	235.3 ± 5.5###	0.09 ± 0.002###	264.4 ± 4.1###	4

[†]Values are expressed as mean ± SEM of parameter estimates from individual fits derived from three experiments carried out in triplicate. The values for basal conditions were 103.6 ± 10.3, 181.8 ± 12.2, and 99.45 ± 15.9 pM in prenatal, postnatal, and adult human brain cortical membranes, respectively. Δ indicates the potency changes calculated as antilog ($pEC_{50GTP\gamma S+Cch}$ - $pEC_{50GTP\gamma S}$), where these are significantly different from GTPγS alone ($P < 0.05$). W22–35, 22–35 weeks of gestational age; Y4–13, 4–13 year old child; Y22–25, 22–25 year old adult.

***$P < 0.001$ prenatal vs. postnatal and adult (unpaired two-tailed Student's *t*-test).

###$P < 0.001$ postnatal vs. adult (unpaired two-tailed Student's *t*-test).

membranes from fetal, child, and adult cases. An aliquot of pooled standard cortical membrane proteins from an adult human brain (Y23) was run in one lane of every gel to minimize interassay variation, and the optical density units obtained from each sample were normalized against those from the pooled cortical standard. Previously, to test whether nonspecific effects (i.e., total protein content per well) could occur under our experimental conditions, immunostaining for the cytoskeletal protein actin was performed in all human brain cases. As shown in Figure 2, analysis of the immunoblots revealed no statistical difference in actin immunolabeling levels among the three aged groups. Subsequently, immunostaining for the cytoskeletal protein actin was performed in all blots to test the consistency of total protein loaded in each gel lane as described in Materials and Methods. The relative values of the levels of $G\alpha_{q/11}$ subunits are presented in Figure 3. Statistical differences in the levels of the $G\alpha_{q/11}$ subunits were not found. To investigate fully the PLC-β isoform composition of human cortical membranes during development, PLC-β_{1-4} isoforms were analyzed. As expected, the PLC-βs examined were found to be expressed in all membrane preparations. However, significantly higher levels of each of the four PLC-β isoforms studied were found in child compared with fetal cortical membranes (Figs. 4, 5).

To establish whether the decreased carbachol-mediated functional responses in fetal cortical membranes were associated with specific changes in the density of muscarinic receptors, we assessed changes in the density and affinity of the total population of muscarinic receptors as well as of sites of high and low affinity for pirenzepine over the course of development. Specific binding of [^{3}H]QNB to cortical membranes from fetal, child, and adult human brains was saturable over the range of concentrations tested. The Scatchard plots of the binding data were linear in all cases, indicating that the labeled ligand was bound to a homogeneous population of sites. The K_d values for [^{3}H]QNB were similar for the three age groups, being approximately 40–50 pM. In contrast, a statistically significant increase in the number of muscarinic receptors (B_{max}) was found in child and adult preparations compared with fetal membranes (Table III). Because [^{3}H]QNB potentially recognized all five muscarinic receptor subtypes equally, a second series of receptor binding studies was performed with pirenzepine. Significant changes in the proportion of high and low pirenzepine affinity sites were found as measured by their displacement of [^{3}H]QNB (Table III). At prenatal stages, the ratio of high- to low-affinity pirenzepine binding sites was approximately 1:1, whereas, at infant and adult stages, this ratio was approximately 2:1 and 3:1, respectively (Table III).

We next evaluated the relative abundance of the $M_{1,3,5}$ muscarinic receptors proteins by means of Western blotting and compared these levels as a function of age (Figs. 6–8). The $M_{1,3,5}$ muscarinic receptor proteins were found to be present in the frontal cortex of the adult human brain. The band sizes observed for M_1 (52 kDa), M_3 (55 kDa), and M_5 (45 kDa) by SDS-PAGE under reducing conditions were consistent with the molecular weight of the nonglycosylated monomeric proteins. In all cases, only a weak band of protein was detectable in extracts from the frontal cortex of the fetal human brain. The M_1 receptor was expressed earliest, and its level was approximately 5% of the adult level by the end of 40 weeks and about 80% of the adult level by the end of infant period (13 years of age). The level of the M_3 subtype was

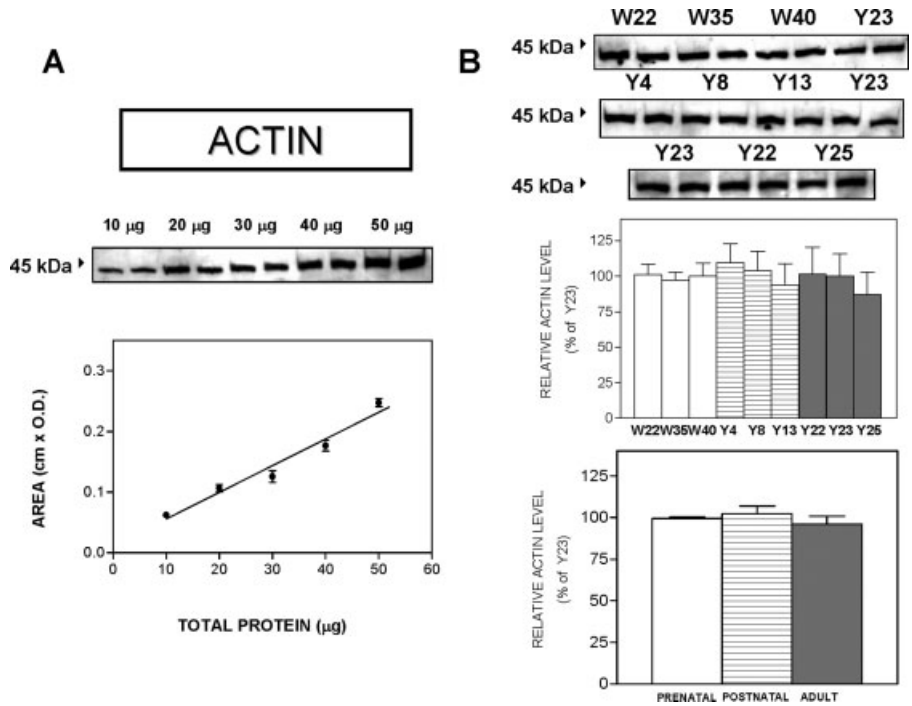

Fig. 2. Levels of actin (cytoskeleton protein) immunoreactivity in human cortical membranes. **A:** Representative immunoblotting with incremental amounts of total protein ranging from 10 to 50 µg. Standard curve for actin in crude human cerebral cortex membranes from which the correlation coefficient ($r = 0.9611$) was obtained by linear regression analysis. **B:** Upper panel: Representative Western blots of actin in duplicate samples of human cortical membranes from prenatal (W22, W35, and W40), postnatal (Y4, Y8, and Y13), and adult (Y22, Y23, and Y25) groups. W, weeks of gestational age; Y, years old. Quantification of immunoblotting. Differences in the levels of actin were assessed in the separate immunoblot experiments carried out in duplicate, using 16 µg of cortical membrane protein. Data are expressed as the mean ± SEM percentage of the corresponding adult human brain case (Y23) from $n = 15$ experiments. Lower panel: Mean ± SEM of values for each individual belonging to the prenatal, postnatal, and adult groups. Statistical differences in the levels of actin were not found to be associated with development. Therefore, this cytoskeleton protein allowed us to exclude potential variations in total protein content per well among fetal, child, and adult cases in Western blot analysis.

approximately 15% of the adult level at the prenatal stage and 70% of the adult level in the child cases. It was not possible to detect the M_5 bands in fetal extracts because of the low signal to background ratio on the films. However, the levels during childhood were approximately 55% of the adult levels.

Subsequently, we examined the responsiveness of PLC to stimulation by calcium to evaluate whether PLC activation involving direct receptor/$G_{q/11}$ protein/PLC-β coupling, as in the case of muscarinic receptor stimulation, was similar to that induced by calcium alone. All PLC isoforms carry out Ca^{2+}-dependent hydrolysis of PIP_2, so raising the calcium concentration directly activates these enzymes. The effect of free Ca^{2+} on [^{3}H]inositol phosphate formation from [^{3}H]PIP_2 is shown in Table IV. Increasing concentrations of free Ca^{2+} stimulated the hydrolysis of [^{3}H]PIP_2 in membranes from the post-mortem human prefrontal cortex. Significant stimulation was obtained with 10 nM Ca^{2+}, and maximal activity was obtained with 10 µM Ca^{2+}, whereas no enzymatic activity was detected in the presence of 3 mM EGTA and no added CaCl2. As shown in Table IV, the half-maximal activation of basal [^{3}H]inositol phosphate formation from [^{3}H]PIP_2, estimated by nonlinear regression analysis, was about 300 nM free Ca^{2+} irrespective of the developmental stage of the preparation. The Ca^{2+} concentration dependence of this response is similar to that reported previously for rat and human brain cortical membranes (Claro et al., 1989; Garro et al., 2001). In addition, the maximal effects of Ca^{2+} were similar for the prenatal, postnatal, and adult groups (Table IV).

Finally, to account for the overall PLC activity measured under calcium-stimulating conditions, the protein levels of PLC-γ and PLC-δ_1 were also examined. No significant differences were found in the levels of expression of PLC-γ in fetal, child, and adult cases (Fig. 9). In contrast, PLC-δ_1 levels were 50% of adult levels during the prenatal stage and equal to the adult level in the postnatal stage (Fig. 9).

DISCUSSION

Evidence from molecular cloning indicates that there are distinct, intronless human genes that encode five muscarinic receptor glycoproteins (Caulfield, 1993; Felder, 1995). Examination of the distribution of the mRNAs

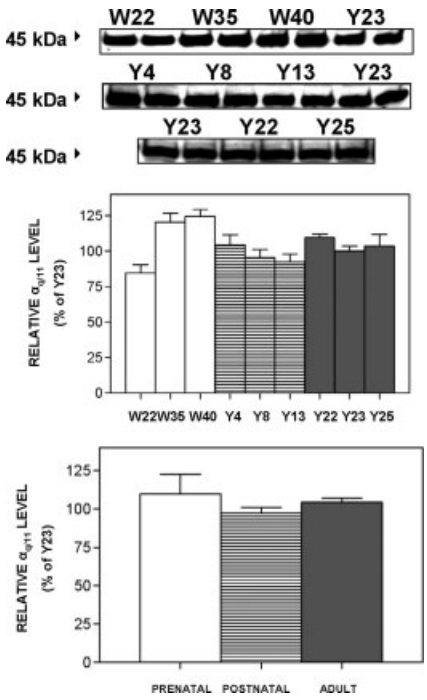

Fig. 3. Quantification of $G\alpha_{q/11}$ subunit immunoreactivity in cortical membranes. Upper panel: Representative Western blots of $G\alpha_{q/11}$ subunits in duplicate samples of cortical membranes from the human cases studied. Abbreviations as in the legend to Figure 2. Middle panel: Differences in the levels of $G\alpha_{q/11}$ were assessed by using 16 μg of protein from cortical membranes from human brain cases. Data are expressed as mean ± SEM percentage with respect to the corresponding adult human brain (Y23) from n = 8 experiments. Lower panel: Mean ± SEM of values from each individual belonging to the prenatal, postnatal, and adult groups. Statistical differences in the levels of the $G\alpha_{q/11}$ subunits were not found to be associated with development.

encoding the five muscarinic receptors has been accomplished by in situ hybridization histochemistry, Northern blot analysis, and quantitative RT-PCR employing receptor-specific nucleic acid probes (Caulfield, 1993; Wei et al., 1994; Krejci and Tucek, 2002). These studies have shown that muscarinic receptor mRNAs have a distinct regional distribution in the mammalian brain, and they have confirmed the presence of muscarinic receptors in brain regions such as the frontal cortex. Moreover, the composition and relative abundance of the entire family of muscarinic receptor proteins in the rat and human brain cortex have been elucidated by radioligand binding studies with tissue homogenates and sophisticated kinetic analyses (Flynn et al., 1997) as well as by immunoprecipitation studies (Wall et al., 1992; Flynn et al., 1995; Tice et al., 1996).

Confirmation of effective coupling of M_1 and M_3 to phosphoinositide-hydrolysis-linked signaling has been reported in native tissues such as the brain cortex both in rat and humans (Forray and El-Fakahany, 1990; Sallés et al., 1993a; Garro et al., 2001). Direct evidence for this pathway of M_5 receptor signaling remains to be established (for comprehensive review see Eglen and Nahorski, 2000). In several respects, the profile of antagonist affinities at the M_5 subtype resembles that determined at the M_3 receptor (Buckley et al., 1989; Dorje et al., 1991; Watson et al., 1999; Eglen and Nahorski, 2000). Therefore, given the similarity in M_3 and M_5 binding profiles, it would be anticipated that M_3 and M_5 fall within the previously defined low-affinity component of pirenzepine binding obtained from functional studies in human brain cortical membranes (Garro et al., 2001) and could reflect an overestimation of the role of the M_3 muscarinic receptor subtype in carbachol-stimulated PLC-β activity.

Information regarding the functional role of a receptor in the CNS can be gleaned from changes in the pattern of expression of that receptor during the course of development. The developmental time course of [^{3}H]QNB binding sites in the human prefrontal cortex reported in the present study is consistent with that previously reported for comparable stages of development (Ravikumar and Sastry, 1985; Perry et al., 1986; Gremo et al., 1987). Thus, binding sites for muscarinic cholinergic agents have been detected during the early stages of development of the human fetal brain (Ravikumar and Sastry, 1985; Flórez et al., 1990; Bar-Peled et al., 1991). In addition, we found significant developmental changes in the proportion of high- and low-affinity pirenzepine binding sites as measured by their displacement of [^{3}H]QNB. Because interpretation of these results in terms of muscarinic receptor subtypes is difficult, we also determined the levels of M_1, M_3, and M_5 muscarinic receptors by Western blot. We found that M_1 muscarinic receptors are expressed very early in the fetal human prefrontal cortex, a situation contrasting with the developmental profile of the M_5 muscarinic receptor. In fact, reliable measures for significant levels of M_5 muscarinic receptors were obtained mostly at postnatal stages. Common to all these development patterns is the fact that carbachol-simulated PLC-β activity in the developing brain differs greatly from that of the adult condition and changes rapidly over the course of development. The present results indicate that carbachol (in the presence of GTPγS) was able to activate PLC-β in all prenatal, postnatal, and adult tissues examined, although the responses were markedly different depending on the stage of development. This finding is consistent with the preceding results regarding the levels of expression of receptors preferentially coupled to phosphoinositide turnover.

The relative lack of responsiveness to GTPγS and carbachol in fetal brain membranes may be due to a low level of expression of $G_{q/11}$ proteins, and/or of muscarinic receptors, and/or of enzymatic activity at early stages of development. Since GTPγS stimulation of PLC-β does not require receptor activation, insufficient G-protein function, which could be due to a decrease in the number and/or sensitivity to GTPγS of $G_{q/11}$ proteins, could explain the weak concentration-response to GTPγS curves at prenatal stages. However, we found by Western blot that $G\alpha_{q/11}$ levels did not change significantly during development, making this unlikely. In addition, it is possi-

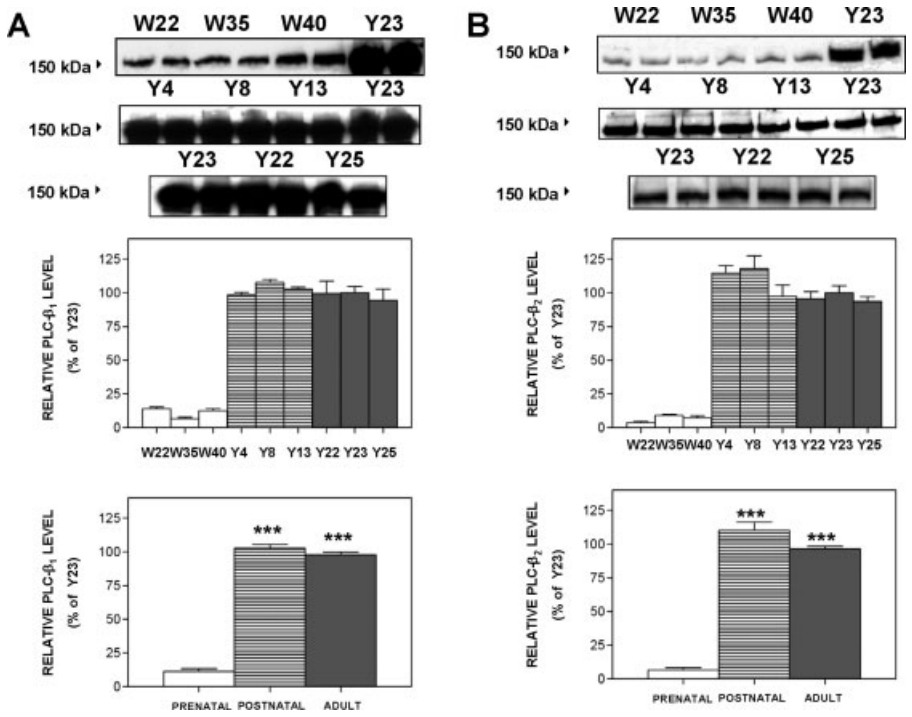

Fig. 4. Quantification of PLC-β_1 (**A**) and PLC-β_2 (**B**) immunoreactivity in cortical membranes. Upper panels: Representative Western blots of PLC-β_1 (A) and PLC-β_2 (B) in duplicate samples of cortical membranes from the human cases studied. Abbreviations as in the legend to Figure 2. Middle panels: Differences in the levels of PLC-β_1 and PLC-β_2 were assessed in separate immunoblot experiments carried out in duplicate, with 6 (A) and 16 (B) μg of protein from cortical membranes from human brain cases. Data are expressed as the mean ± SEM percentage of the corresponding adult human brain case (Y23) from three experiments. Lower panels: Mean ± SEM of values for each individual belonging to the prenatal, postnatal, and adult groups. $^{***}P < 0.001$ prenatal vs. postnatal and adult (unpaired two-tailed Student's *t*-tests). Significantly higher levels of PLC-β_1 and PLC-β_2 were found in child compared with fetal cortical membranes.

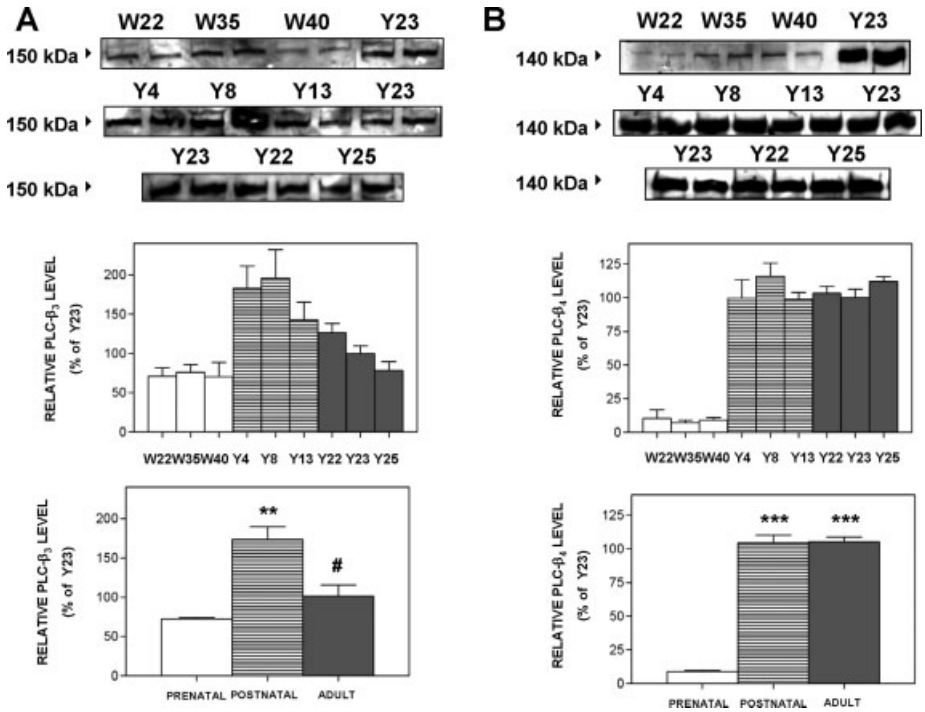

Fig. 5. Quantification of PLC-β_3 (**A**) and PLC-β_4 (**B**) immunoreactivity in cortical membranes. Upper panels: Representative Western blots of PLC-β_3 (A) and PLC-β_4 (B) in duplicate samples of cortical membranes from the human cases studied. Abbreviations as in the legend to Figure 2. Middle panels: Differences in the levels of PLC-β_3 and PLC-β_4 were assessed in separate immunoblot experiments carried out in duplicate, with 16 μg of protein from cortical membranes from human brain cases. Data are expressed as the mean ± SEM percentage of the corresponding adult human brain case (Y23) from three cases. Lower panels: Mean ± SEM of values for each individual belonging to the prenatal, postnatal, and adult groups. $^{***}P < 0.001$ prenatal vs. postnatal and adult; $^{\#}P < 0.05$ postnatal vs. adult (unpaired two-tailed Student's *t*-tests). Once again, significantly higher levels of PLC-β_3 and PLC-β_4 were found in child compared with fetal cortical membranes.

ble that the amount of PLC specifically coupled to G proteins is higher in the post- vs. prenatal brain. In fact, in the rodent CNS, the three structural families (β-, γ-, and δ-) of PLC isoforms present a different developmental pattern of expression, with increased levels of PLC-β isoforms (mRNA and protein) in the mature brain (Yamada et al., 1991; Shimohama et al., 1998; Watanabe et al., 1998). Our Western blot data now extend these findings to the human brain; the dramatically higher levels of expression of the PLC-β_{1-4} isoforms in child compared with fetal brains may underlie the increased responsiveness to GTPγS and carbachol. Moreover, these differences cannot be attributed to nonspecific effects (total protein loaded per well), because the immunolabeling of the cytoskeleton

TABLE III. Parameters of [^{3}H]QNB Binding to Human Cortical Membranes†

	Saturation isotherms of [^{3}H] QNB			Competition of pirenzepine for [^{3}H] QNB binding sites			
Case	B_{max} (fmol/mg)	K_d (pM)	n	$\%R_1$	pK_{i1}	pK_{i2}	n
W22	82.0	50	1				
W35	255.68	40	1	58.2 ± 6.4	8.22 ± 0.10	6.77 ± 0.13	3
W40	199.57	43	1	58.8	8.00	6.60	1
Prenatal	179.2 ± 51.1*	44 ± 3.0		58.6 ± 0.3	8.11 ± 0.11	6.68 ± 0.09	
Y4	427.2 ± 17.6	42 ± 6.0	3	74.1 ± 0.2	7.80 ± 0.07	6.61 ± 0.19	3
Y8	378.4 ± 4.7	43 ± 2.0	3	62.5 ± 0.3	8.05 ± 0.22	6.91 ± 0.37	3
Y13	375.9 ± 41.8	41 ± 7.0	3	70.0 ± 1.1	7.99 ± 0.12	6.74 ± 0.27	3
Postnatal	393.74 ± 16.73	42 ± 0.6		68.9 ± 3.4	7.95 ± 0.08	6.75 ± 0.09	
Y22	410.14 ± 16.3	47 ± 3.0	3	83.7 ± 6.3	7.80 ± 0.05	6.51 ± 0.39	3
Y23	507.76 ± 17.3	43 ± 2.0	4	85.9 ± 2.0	7.79 ± 0.05	6.40 ± 0.31	3
Y25	397.3 ± 12.8	39 ± 5.1	3				
Adult	438.4 ± 34.9	43 ± 2.3		84.8 ± 1.0	7.79 ± 0.01	6.45 ± 0.05	

†Values are expressed as mean ± SEM of parameter estimates from individual fits derived from the indicated number of experiments (n), which were performed in triplicate. pK_{i1} and pK_{i2} correspond to high - and low-affinity components of pirenzepine. $\%R_1$ is the high-affinity fractions of pirenzepine-induced inhibition. A two-site model was retained only if it was significantly better than a one-site model. Abbreviations as in Table I.
*$P < 0.05$ prenatal vs. corresponding postnatal and adult values (unpaired two-tailed Student's *t*-test).

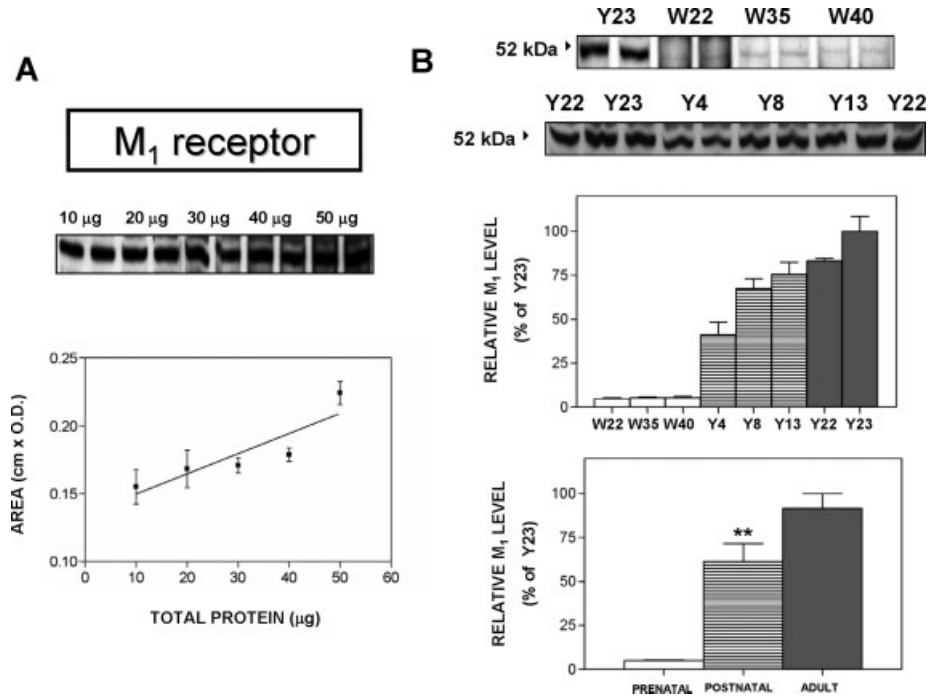

Fig. 6. Levels of muscarinic M_1 receptor immunoreactivity in human frontal cortex membranes. **A:** Elaboration of standard curves for the M_1 muscarinic receptor in crude human cerebral cortex membranes. Upper panel: Immunoblotting using incremental amounts of protein ranging from 10–50 µg. Lower panel: The corresponding standard curves from which the correlation coefficient (r = 0.7912) was obtained by linear regression analysis. **B:** Upper panel: Representative Western blots of the M_1 muscarinic receptor in duplicate samples of human cortical membranes. Abbreviations as in the legend to Figure 2. Middle panel: Quantification of immunoblotting. Differences in the levels of M_1 muscarinic receptors were assessed in the separate immunoblot experiments carried out in duplicate, with 30 µg of cortical membrane protein. Data are expressed as the mean ± SEM percentage of the corresponding adult human brain case (Y23). Lower panel: Mean ± SEM of values for each individual belonging to the prenatal, postnatal and adult (Y22, Y23) groups. **$P < 0.01$ prenatal vs. postnatal (unpaired two-tailed Student's *t*-tests). By the end of the prenatal period (40 weeks), the level of the M_1 receptor was approximately 5% of that of the adult level but was about 80% of that of the adult level by the end of infant period (13 years of age).

protein actin, used as an internal control, was similar among the three age group studied. Hence, although much more work will have to be done to establish the role that the relative amounts of muscarinic receptors/G proteins/PLC-βs play during development and to explain why responses to carbachol, as defined in cell-free systems, are weak in prenatal stages of development, our data indicate that the rate of phosphoinositide hydrolysis may be

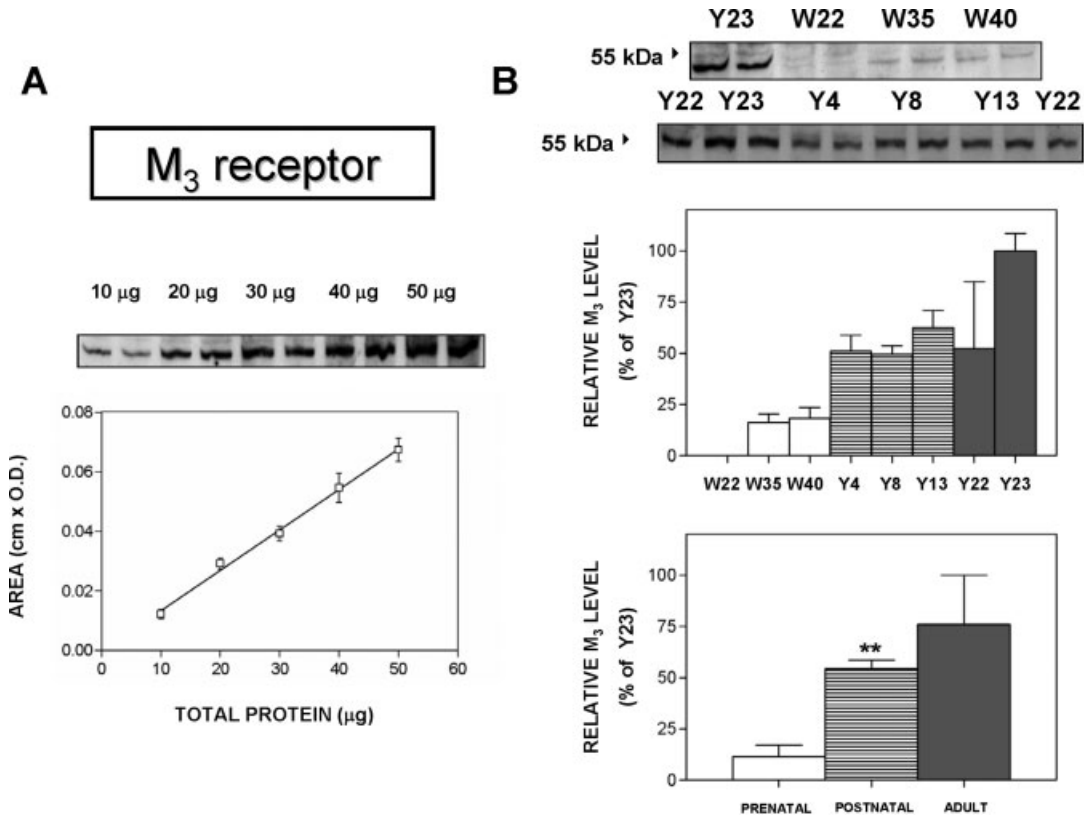

Fig. 7. Levels of muscarinic M_3 receptor immunoreactivity in human frontal cortex membranes. **A:** Elaboration of the standard curve for the M_3 muscarinic receptor in crude human cerebral cortex membranes. Upper panel: Immunoblotting with incremental amounts of protein ranging from 10 to 50 µg. Lower panel: The corresponding standard curves from which the correlation coefficient (r = 0. 9955) was obtained by linear regression analysis. **B:** Upper panel: Representative Western blots of the M_3 muscarinic receptor in duplicate samples of human cortical membranes. Abbreviations as in the legend to Figure 2. Middle panel: Quantification of immunoblotting. Differences in the levels of M_3 muscarinic receptors were assessed in three separate immunoblot experiments carried out in duplicate, with 30 µg of protein from human cortical membranes. Data are expressed as the mean ± SEM percentage of the adult human brain case (Y23). Lower panel: Mean ± SEM of values for each individual belonging to the prenatal, postnatal, and adult (Y22, Y23) groups. $^{**}P < 0.01$ prenatal vs. postnatal (unpaired two-tailed Student's *t*-tests). The level of the M_3 subtype was approximately 15% of the adult level at the prenatal stage and 70% of the adult level in the child cases.

limited during fetal stages of development by low levels of expression of PLC-βs and of M_1, M_3, and M_5 muscarinic receptors rather than by reduced availability of $G\alpha_{q/11}$ subunits.

Nevertheless, the higher functional responses measured in the child brain in comparison with the adult brain (see also Garro et al., 2001), in spite of similar levels of expression of muscarinic receptors/$G\alpha_{q/11}$ subunits/PLC-β_{1-4} isoforms remains an intriguing issue. A similar ontogenic developmental profile has been reported for the rat brain, for which several groups have shown that muscarinic receptor-stimulated phosphoinositide metabolism is higher in the neonatal rat brain (in which the brain growth spurt is taking place; Dobbing and Sands, 1979) than in the corresponding adult brain (Balduini et al., 1987; Heacock et al., 1987; Rooney and Nahorski, 1987). Decreased carbachol-stimulated PLC-β activity in cortical membranes from adult brains may be due to an uncoupling of muscarinic receptors from the corresponding G-proteins, as indicated by the difference in size of the shift induced by carbachol in the GTPγS concentration-response curves. However, the classical view that G-protein-coupled receptor systems are one-dimensional cannot readily account for these observations, and, as discussed by Ostrom (2002), an additional dimension must be incorporated into this conceptual model. A more contemporary idea is that cellular plasma membranes do not uniformly express G-protein-coupled-receptor-effector molecules, but, rather, they do so in specific membrane microdomains (i.e., compartmentation; see Ostrom et al., 2000; Ostrom 2002). Thus, it is possible that muscarinic receptors, $G_{q/11}$ proteins, and/or PLC-βs, though present at similar levels in child and adult human cortical membranes, undergo alterations in their colocalization that determine muscarinic receptor coupling efficiency to PLC-β. Nevertheless, it must be remembered that activation of phospholipase C-β by GTPγS and carbachol in brain membranes is seen in the presence of 1 mM deoxycholate (Claro et al., 1989),

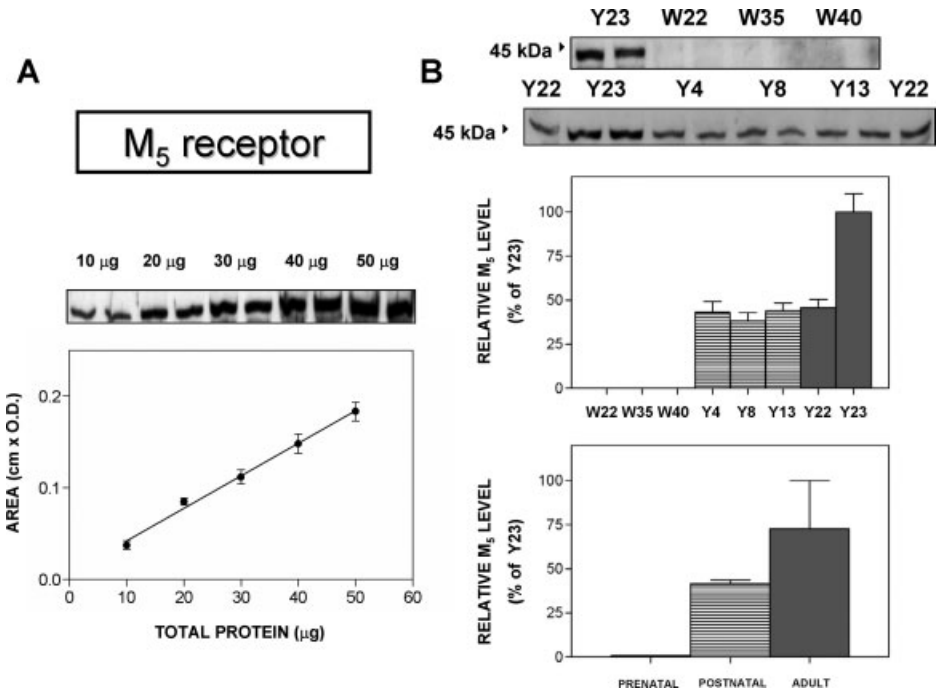

Fig. 8. Levels of muscarinic M_5 receptor immunoreactivity in human frontal cortex membranes. **A:** Standard curves for the M_5 muscarinic receptor subtype in crude human cortical membranes. Upper panel: Immunoblotting using incremental amounts of protein ranging from 10 to 50 μg. Lower panel: The corresponding standard curves from which the correlation coefficient (r = 0. 9938) was obtained by linear regression analysis. **B:** Upper panel: Representative Western blots of the M_5 muscarinic receptor subtype in duplicate samples of human cortical membranes. Abbreviations as in the legend to Figure 2. Middle panel: Quantification of immunoblotting. Differences in the levels of M_5 muscarinic receptors were assessed in three separate immunoblot experiments carried out in duplicate, with 30 μg of protein from human cortical membranes. Data are expressed as the mean ± SEM percentage of the corresponding adult human brain case (Y23). Lower panel: Mean ± SEM of values for each individual belonging to the prenatal postnatal and adult (Y22, Y23) groups. It was not possible to detect the M_5 bands in fetal membranes because of the low signal-to-background ratio. However, M_5 levels during childhood were approximately 55% of those detected in adult preparations.

and it is now known that under these conditions spatial segregation would be preserved.

It is also conceivable that enhanced stimulation of PIP_2 metabolism in child tissue may be due to a lack of inhibitory feedback by G-protein-coupled receptor kinases (GRKs) and/or other kinases. In fact, in the rat brain, the expression of GRK2 and β-arrestin-1 was only about 45–70% and 30%, respectively, of the adult levels by the end of first postnatal week (Penela et al., 2000). Both the M_1 and the M_3 muscarinic receptor subtypes are phosphorylated by GRK2 in an agonist-dependent manner (Debburman et al., 1995; Haga et al., 1996). It has been suggested that the phosphorylation of G-protein-coupled receptors greatly reduces their signal transduction functionality (for review see Krupnick and Benovic, 1998). In addition, recent studies have shown that certain GRKs can suppress the interaction between GPCRs and G-proteins in a phosphorylation-independent manner. In this respect, Carman et al. (1999) demonstrated that GRK2 and GRK3 bind avidly to activated $G\alpha_{q/11}$ to inhibit Ins(1,4,5)-P_3 generation.

The functional differences observed in agonist-stimulated PLC-β activity could be attributed to demographic characteristics of the subjects analyzed, such as gender or post-mortem interval. There currently are no data to indicate that gender influences muscarinic receptor signalling via PLC in the developing human brain; nevertheless, we cannot exclude this possibility because of the limited number of samples analyzed in this study. In con-

TABLE IV. Concentration-Effect Curves for Calcium on PLC Activity in Human Brain Cortical Membranes*

Human case	EC_{50} (nM)	E_{max} (pM)
W22	229.5 ± 54.2	549.8 ± 35.5
W35	569.6 ± 102	1,244.1 ± 139.2
W40	459.0 ± 33.4	945.6 ± 117.6
Prenatal	419.4 ± 100.2	913.2 ± 201.1
Y4	257.6 ± 12.2	1,138.16 ± 23.9
Y8	239.8 ± 11.4	1,656.9 ± 46.1
Y13	261.8 ± 12.0	1,223.5 ± 22.6
Postnatal	253.1 ± 6.7	1,339.5 ± 160.6
Y22	235.8 ± 23.7	732.5 ± 108.1
Y23	298.4 ± 14.5	1,113.8 ± 6.1
Y25	267.1 ± 18.9	1,009.1 ± 49.9
Adult	267.1 ± 18.1	951.8 ± 113.7

*Values are expressed as mean ± SEM of parameter estimates from individual fits derived from n = 3 experiments each run in triplicate. Abbreviations as in Table I.

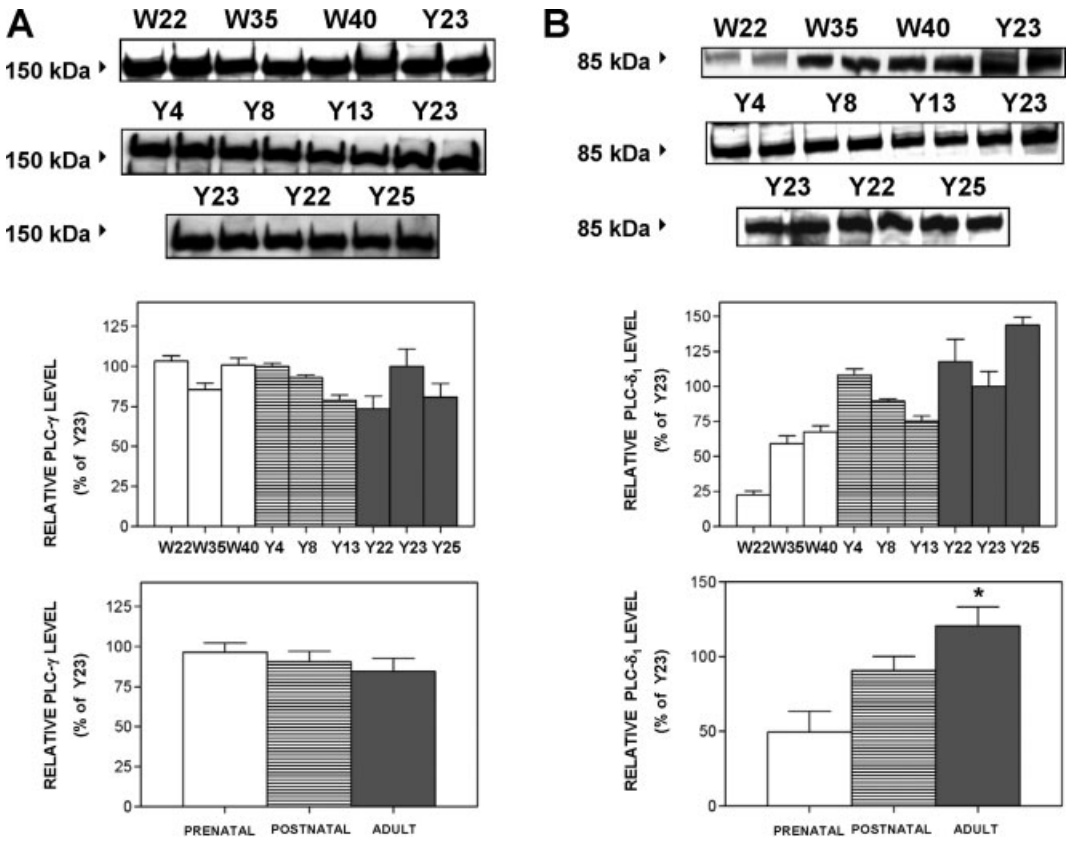

Fig. 9. Quantification of immunoblotting. **A:** Upper panel: Representative Western blots of PLC-γ isoforms in duplicate samples of cortical membranes from the human cases studied. Abbreviations as in the legend to Figure 2. Middle panel: Quantification of immunoblotting. Differences in the levels of PLC-γ were assessed in three separate immunoblot experiments carried out in duplicate, with 16 μg of protein from cortical membranes from human brain cases. Data are expressed as the mean ± SEM percentage of the corresponding adult human brain case (Y23) from three experiments. Lower panel: Mean ± SEM of values for each individual belonging to the prenatal (W22, W35, and W40), postnatal (Y4, Y8, and Y13), and adult (Y22, Y23, and Y25) groups. **B:** Upper panel: Representative Western blots of PLC-δ_1 isoforms in duplicate samples of cortical membranes from the human cases studied. Middle panel: Quantification of immunoblotting. Differences in the levels of PLC-δ_1 were assessed in three separate immunoblot experiments carried out in duplicate, with 16 μg of protein from cortical membranes from human brain cases. Data are expressed as the mean ± SEM percentage of the corresponding adult human brain case (Y23) from three experiments. Lower panel: Mean ± SEM of values for each individual belonging to the prenatal (W22, W35, and W40), postnatal (Y4, Y8, and Y13), and adult (Y22, Y23, and Y25) groups. *$P < 0.05$ prenatal vs. adult (unpaired two-tailed Student's *t*-test).

trast, controversial data have been published on the influence of the post-mortem interval on each of the proteins analyzed (either functionally or in terms of expression level) in this study. On the one hand, the basal activity of PLC and its stimulation by GTPγS or carbachol in membranes from human prefrontal cortex samples were unaffected by post-mortem delays ranging from 5 to 21 hr (Jope et al., 1994; Greenwood et al., 1995). Furthermore, expression levels of PLC isoforms determined by Western blot analysis were unaffected by varying post-mortem intervals (Greenwood et al., 1995). However, post-mortem stability of stimulated phosphoinositide hydrolysis occurred in spite of the fact that there was a significant correlation between decreased levels of $G\alpha_q$ and increased post-mortem interval (Greenwood et al., 1995). These contradictory findings may be the result of either $G\alpha_q$ being present in excess or of the small degree of decline of $G\alpha_q$ levels (Greenwood et al., 1995; Li et al., 1996). Our results also suggest that the rate of phosphoinositide hydrolysis might not be limited by $G\alpha_{q/11}$ levels in human brain cortical membranes (López de Jesús et al., 2006; present study). On the other hand, a post-mortem-delay-mediated decrease in GTPγS and carbachol-stimulated phosphoinositide metabolism has been reported for human brain cortical membranes (Garro et al., 2004). The effect of post-mortem delay on the density of muscarinic receptors is also unclear; reports of no changes (Rodríguez-Puertas et al., 1996), a slight reduction (Whitehouse et al., 1984), and significant decreases (Cortés et al., 1987) have been published. In any event, the potential impact of post-mortem delay on the results reported here would not appear to affect our conclusions, at least qualitatively, in particular because those cases with longer post-mortem delay are precisely those with higher functional responses

and higher protein expression levels of receptors and effectors (child ≥ adult > fetal).

Finally, we examined the responsiveness of PLC to stimulation by calcium and did not detect significant differences in the maximal effects of calcium among the various developmental stages examined. Thus, the developmental pattern of carbachol-stimulated enzymatic activity is very different from that stimulated by calcium. However, it must be remembered that all PLC isoenzyme activities are calcium dependent (Rhee and Bae, 1997); therefore, all the PLC isoenzymes present in membranes are likely to contribute to the total PLC activity measured. Because low levels of PLCβ isoforms are associated with the prenatal cortex, other PLC members must contribute to the robust calcium response. Our Western blot results indicate that PLCγ and, to a lesser extent, PLCδ$_1$ make important contributions to calcium-stimulated PLC activity in fetal cortical membranes.

Overall, the present results demonstrate that PLCβ, -γ, and -δ isoforms are present in the fetal, child, and adult prefrontal cortex of the human brain, increasing levels being found over development for PLC-β and δ, whereas PLCγ levels at early developmental stages were similar to those in the adult. PLC-γ has been suggested to be involved especially in cellular division and growth during rodent CNS histogenesis, whereas PLC-β and PLC-δ seem to be involved in early synaptogenesis and establishment of the neural network (Yamada et al., 1991; Shimohama et al., 1998) by means of regulating the differentiation and maturation of neurons in the developing brain. Our results also suggest that more efficient inositol phosphate production by guanine nucleotides and carbachol in the postnatal vs. prenatal brain is achieved by up-regulating the expression of receptors ($M_{1,3,5}$ subtypes) and effectors (PLC-β_{1-4}) rather than by altering the levels of $G\alpha_{q/11}$ subunits. The developing human brain thus provides a useful longitudinal model that lends itself particularly well to tackling the problem of how muscarinic receptor coupling to phosphoinositide breakdown is regulated. Therefore, these studies could be of critical importance in understanding better the substrate underlying both congenital and acquired neurodevelopmental diseases of the CNS.

ACKNOWLEDGMENTS

This work was supported by grants from the Spanish Ministry of Science and Technology (MCYT; SAF98-0064 and SAF01-0651) to A.P. and J.S. and the Basque Government (ETORTEK) to J.S. In addition, I.R.A. received a fellowship from the Euskal Herriko Unibertsitatea/University of the Basque Country. Finally, the authors thank the agency ACTS (http://www.euskalnet.net/acts) for having improved the English in this paper.

REFERENCES

Aguilar JS, Lunt GG. 1985. Muscarinic receptor sites in human fetal brain. Neurochem Int 7:509–514.

Balduini W, Murphy SD, Costa LG. 1987. Developmental changes in muscarinic receptor-stimulated phosphoinositide metabolism in rat brain. J Pharmacol Exp Ther 241:421–427.

Bar-Peled O, Israeli M, Ben-Hur H, Hoskins I, Groner Y, Biegon A. 1991. Developmental pattern of muscarinic receptors in normal and Down's syndrome fetal brain: an autoradiographic study. Neurosci Lett 133:154–158.

Buckley NJ, Bonner TI, Buckley CM, Brann MR. 1989. Antagonist binding properties of five cloned human muscarinic receptor subtypes. Mol Pharmacol 35:469–476.

Cali JJ, Zwaagstra JC, Mons N, Cooper DMF, Kupinski J. 1994. Type VIII adenylyl cyclase: a Ca^{2+}/calmodulin-stimulated enzyme expressed in discrete regions of rat brain. J Biol Chem 269:12190–12195.

Candura SM, Tonini M, Baiardi P, Manzo L, Costa LG. 1995. Heterogeneity of cholinergic muscarinic receptors coupled to phosphoinositide metabolism in immature rat brain. Brain Res Dev Brain Res 86:134–142.

Carman CV, Parent JL, Day PW, Pronin AN, Sternweis PM, Wedegaertner PB, Gliman AG, Benovic ,JL, Kozasa T. 1999. Selective regulation of $G\alpha_{q/11}$ by RGS domain in the G protein-coupled kinase, GRK2. J Biol Chem 274:34483–34492.

Caulfield MP. 1993. Muscarinic receptors—characterization, coupling and function. Pharmacol Ther 58:319–379.

Claro E, Wallace MA, Lee HM, Fain JN. 1989. Carbachol in the presence of guanine 5′-O-(3-thiotriphosphate) stimulates the breakdown of exogenous phosphatidylinositol 4,5-biphosphate, phosphatidylinositol 4-phosphate, and phosphatidylinositol by rat brain membranes. J Biol Chem 264:18288–18295.

Cortés R, Probst A, Palacios JM. 1987. Quantitative light microscopic autoradiographic localization of cholinergic muscarinic receptors in the human brain: forebrain. Neuroscience 20:65–107.

Crews FT, Kurian P, Freund G. 1994. Cholinergic and serotonergic stimulation of phosphoinositide hydrolysis is decreased in Alzheimer's disease. Life Sci 55:1993–2002.

Debburman SK, Kunapuli P, Benovic JL, Hosey M. 1995. Agonist-dependent phosphorylation of human muscarinic receptors in Spodoptera frugiperda insect cell membranes by G protein-coupled receptor kinases. Mol Pharmacol 47:224–233.

Dobbing J, Sands J. 1979. Comparative aspects of the brain growth spurt. Early Hum Dev 3:79–83.

Dorje F, Wess J, Lambrecht G, Tacke R, Mutschler E, Brann MR. 1991. Antagonist binding profiles of five cloned muscarinic receptor subtypes. J Pharmacol Exp Ther 256:727–733.

Eglen RM, Nahorski SR. 2000. The muscarinic M5 receptor: a silent or emerging subtype? Br J Pharmacol 130:13–21.

Egozi Y, Sokolovsky M, Schefter E, Blatt Y, Zakut H, Matzkel A, Soreq H. 1986. Divergent regulation of muscarinic binding sites and acetylcholinesterase in discrete regions of the developing human fetal brain. Cell Mol Neurobiol 6:55–70.

Felder CC. 1995. Muscarinic acetylcholine receptors: signal transduction through multiple effectors. FASEB J 9:619–625.

Fleming WW, Westfall DP, De La Lande IS, Jellett LB. 1972. Long-normal distribution of equieffective doses of norepinephrine and acetylcholine in several tissues. J Pharmacol Exp Ther 181:339–345.

Florez J, Del Arco C, González A, Pascual J, Pazos A. 1990. Autoradiographic studies of neurotransmitter receptors in the brain of newborn infants with Down's syndrome. Am J Med Genet 7:301–305.

Flynn DD, Ferrari-DiLeo G, Mash DC, Levey AI. 1995. Differential regulation of molecular subtypes of muscarinic receptors in Alzheimer's disease. J Neurochem 64:1888–1891.

Flynn DD, Reever CM, Ferrari-DiLeo G. 1997. Pharmacological strategies to selectively label and localize receptor subtypes. Drug Dev Res 40:104–116.

Forray C, El-Fakahany EE. 1990. On the involvement of multiple muscarinic receptor subtypes in the activation of phosphoinositide metabolism in rat cerebral cortex. Mol Pharmacol 37:893–902.

Garro MA, López De Jesús M, Ruiz de Azúa I, Callado LF, Meana JJ, Sallés J. 2001. Regulation of phospholipase Cβ activity by muscarinic acetylcholine and 5-HT_2 receptors in crude and synaptosomal membranes from human cerebral cortex. Neuropharmacology 40:686–695.

Garro MA, López de Jesús M, Ruiz de Azúa I, Callado LF, Meana JJ, Sallés J. 2004. Differential post-mortem delay effect on agonist-mediated phospholipase Cβ activity in human cortical crude and synaptosomal membranes. Neurochem Res 29:1453–1457.

Greenwood AF, Powers RE, Jope RS. 1995. Phosphoinositide hydrolysis, Gαq, phospholipase C, and protein kinase C in post mortem human brain: effects of post mortem interval, subject age and Alzheimer's disease. Neuroscience 69:125–138.

Gremo F, Palomba M, Marchisio AM, Marcello C, Mulas ML, Torelli S. 1987. Heterogeneity of muscarinic receptor in the developing human fetal brain: regional distribution and characterization. Early Hum Dev 15:165–177.

Haga K, Kameyama K, Haga T, Kikkawa U, Shiozaki K, Uchiyama H. 1996. Phosphorylation of human m1 muscarinic acetylcholine receptors by G protein-coupled receptor kinase 2 and protein kinase C. J Biol Chem 271:2776–2782.

Heacock AM, Fisher SK, Agranoff BW. 1987. Enhanced coupling of neonatal muscarinic receptors in rat brain to phosphoinositide turnover. J Neurochem 48:1904–1911.

Hohmann CF, Berger-Sweeney JB. 1998. Cholinergic regulation of cortical development and plasticity. New twists to an old story. Prespect Dev Neurobiol 5:401–425.

Jope RS, Song L, Powers R. 1994. Agonist-induced, GTP-dependent phosphoinositide hydrolysis in post-mortem human brain membranes. J Neurochem 62:180–186.

Krejci AE, Tucek S. 2002. Quantification of mRNAs for M1 to M5 subtypes of muscarinic receptors in heart and brain cortex. Mol Pharmacol 61:1267–1272.

Krupnick JG, Benovic JL. 1998. The role of receptor kinases and arrestins in a G-protein-coupled receptor regulation. Annu Rev Pharmacol Toxicol 38:289–319.

Larocca JN, Rodriguez-Gabin AG, Rashbaum WK, Weidenheim KM, Lyman WD. 1994. Muscarinic receptor-dependent activation of phospholipase C in the developing human fetal central nervous system. Brain Res 653:9–15.

Li X, Greenwood AF, Powers R, Jope RS. 1996. Effects of post-mortem interval, age and Alzheimer's disease on G-proteins in human brain. Neurobiol Aging 17:115–122.

López de Jesús M, Zalduegui A, Ruiz de Azúa I, Callado LF, Meana JJ, Sallés J. 2006. Levels of G-protein $\alpha_{q/11}$ subunits and phospholipase C-β_{1-4}, -γ, and -δ_1 isoforms in post-mortem human brain caudate and cortical membranes: potential functional implications. Neurochem Int (in press).

Ostrom RS. 2002. New determinants of receptor-effector coupling: trafficking and compartmentation in membrane microdomains. Mol Pharmacol 61:473–476.

Ostrom RS, Post SR, Insel PA. 2000. Stoichiometry and compartmentation in G protein-coupled receptor signaling: implications for therapeutic interventions involving Gs. J Pharmacol Exp Ther 294:407–412.

Penela P, Alvarez-Doblado M, Muñoz A, Mayor F. 2000. Expression patterns of the regulatory proteins G protein-coupled receptor kinase 2 and beta-arrestin 1 during rat postnatal brain development: effect of hypothyroidism. Eur J Biochem 267:4390–4396.

Perry EK. 1986. The cholinergic hypothesis-ten years on. Br Med Bull 61:473–476.

Perry EK, Smith CJ, Atack JR, Candy JM, Johnson M, Perry RH. 1986. Neocortical cholinergic enzyme and receptor activities in the human fetal brain. J Neurochem 47:1262–1269.

Ravikumar BV, Sastry PS. 1885. Muscarinic cholinergic receptors in human fetal brain: characterization and ontogeny of [^{3}H]quiniclidinyl benzilate binding sites in frontal cortex. J Neurochem 44:240–246.

Rhee SG, Bae YS. 1997. Regulation of phosphoinositide-specific phospholipase C isoenzymes. J Biol Chem 272:15045–15048.

Rodríguez-Puertas R, Pascual J, Pazos A. 1996. Effects of freezing storage time on the density of muscarinic receptors in the human postmortem brain: an autoradiographic study in control and Alzheimer's disease brain tissues. Brain Res 728:65–71.

Rooney TA, Nahorski SR. 1987. Postnatal ontogeny of agonist depolarization-induced phosphoinositide hydrolysis in rat cerebral cortex. J Pharmacol Exp Ther 243:333–341.

Rooney TA, Nahorski SR. 1989. Developmental aspects of muscarinic-induced inositol polyphosphate accumulation in rat cerebral cortex. Eur J Pharmacol Mol Pharmacol Sect 172:425–434.

Ruiz de Azúa I, Lumbreras MA, Zalduegui A, Baamonde C, Dierssen M, Flórez J, Sallés J. 2001. Reduced phospholipase C-β activity and isoform expression in the cerebellum of Ts65Dn mouse: a model of Down syndrome. J Neurosci Res 66:540–550.

Sallés J, Wallace MA, Fain JN. 1993a. Differential effects of alkylating agents on the multiple muscarinic receptor subtypes linked to activation of phospholipase C by carbachol in rat brain cortical membranes. J Pharmacol Exp Ther 264:521–529.

Sallés J, Wallace MA, Fain JN. 1993b. Modulation of the phospholipase C activity in rat brain cortical membranes by simultaneous activation of distinct monoaminergic and cholinergic muscarinic receptors. Brain Res Mol Brain Res 20:111–117.

Sallés J, López de Jesús M, Goñi O, Fernandez-Teruel A, Driscoll P, Tobeña A, Escorihuela RM. 2001. Transmembrane signaling through phospholipase C in cortical and hippocampal membranes of psychogenetically selected rat lines. Psychopharmacology 154:115–125.

Shimohama S, Sumida Y, Fujimoto S, Matsuoka Y, Taniguchi T, Takenawa T, Kimura J. 1998. Differential expression of rat brain phospholipase C isoenzymes in development and aging. Biochem Biophys Res Commun 243:210–216.

Tice MAB, Hashemi T, Taylor LA, McQuade RD. 1996. Distribution of muscarinic receptor subtypes in rat brain from postnatal to old age. Brain Res Dev Brain Res 92:70–76.

Wall SJ, Yasuda RP, Li M, Ciesla W, Wolfe BB. 1992. The ontogeny of m1–m5 muscarinic receptor subtypes in rat forebrain. Brain Res Dev Brain Res 66:181–185.

Wallace MA, Claro E. 1990. Comparison of serotoninergic to muscarinic cholinergic stimulation of phosphoinositide-specific phospholipase C in rat brain cortical membranes. J Pharmacol Exp Ther 255:1296–1300.

Wallace MA, Claro E. 1993. Transmembrane signaling through phospholipase C in human brain membranes. Neurochem Res 18:139–145.

Watanabe M, Nakamura M, Sato K, Kano M, Simon MI, Inoue Y. 1998. Patterns of expression for the mRNA corresponding to the four isoforms of phospholipase Cβ in mouse brain. Eur J Neurosci 10:2016–2025.

Watson N, Daniels DV, Ford APDW, Eglen RM, Hedge SS. 1999. Comparative pharmacology of recombinant M3 and M5 muscarinic receptors expressed in CHO-K1 cells. Br J Pharmacol 127:590–596.

Wei J, Walton EA, Milici A, Buccafusco JJ. 1994. m1–m5 muscarinic receptor distribution in rat CNS by RT-PCR and HPLC. J Neurochem 63:815–821.

Whitehouse PJ, Lynch D, Kuhar MJ. 1984. Effects of post-mortem delay and temperature on neurotransmitter receptor binding in a rat model of the human autopsy process. J Neurochem 43:553–559.

Yamada M, Mizuguchi M, Rhee SG, Kim SU. 1991. Development changes of three phosphoinositide-specific phospholipase C isoenzymes in the rat nervous system. Brain Res Dev Brain Res 59:7–16.