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REGULATION OF C-MYC PROTEIN EXPRESSION IN THE DEVELOPING RAT CEREBELLUM BY PHOSPHOINOSITIDE TURNOVER

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SUMMARY: Using developing rat cerebellum, we examined the correlation between the turnover of phosphoinositide (PI) and c-myc expression. The ^{32}P incorporation into polyphosphoinositides changed remarkably with advancing age. It reached a maximum value on PND 7, and then decreased gradually until PND 14. Immunostaining by anti-PIP2 antibody changed in parallel. The expression of c-myc mRNA was also changed developmentally, showing a peak on PND 7; whereas c-MYC protein expression showed a peak on PND 10. Together, these results suggest that c-myc expression is regulated by PI turnover during the early developing stage of rat cerebellum.

Previously we reported the postnatal development of the Ca^{2+}-mobilization system in the cerebellar cortex by measuring enhanced Ca^{2+} concentration under quisqualate (QA) stimulation and by immunohistochemical examination of phosphatidylinositol 4,5-bisphophate (PIP2) and inositol 1,4,5-trisphosphate (IP3) binding protein in rat cerebellum (1). In the report, we showed that the change in Ca response by QA stimulation must be due to the change in the PI turnover rate in developing rat cerebellum, using anti-PIP2 antibody (Ab).

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Abbreviations used: PND - postnatal day; Ab - antibody; IEG - immediate early genes; QA - quisqualate; PI - phosphoinositide; PA - phosphatidic acid; PIP - phosphatidylinositol 4-monophosphate; PIP2 - phosphatidylinositol 4,5-bisphosphate; IP3 - inositol 1,4,5-trisphosphate; PKC - protein kinase C; PLC -phospholipase C.

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In order to establish the cause of this developmental change in phosphoinositide (PI) turnover rate, we examined the expression of immediate early genes (IEG), because of the correlation between PI turnover and c-myc expression in the human renal cancer cell line (2). Recently, proto-oncogene c-myc was found to be expressed during proliferation and/or differentiation of neurons (3-5). Although there are several exceptions, the c-myc expression was thought to correlate with cell growth during embryogenesis in other studies (4, 6). Since the c-myc gene was established as being the cellular homologue of the retroviral v-myc gene (7), the structure and expression of the c-myc gene, especially in cancer cells, has been extensively studied (8-12). Nevertheless, the function of the c-MYC protein and the regulation of its expression remains unclear.

In this paper, we examined the developmental change in c-myc expression and that in c-MYC protein by Northern blotting and in situ hybridization and by immunoblotting with anti-c-MYC Ab, respectively. We found that changes in c-myc expression were in parallel with ^{32}P incorporation into polyphosphoinositides, and that both of them preceded the change in c-MYC protein until PND 14. Since the PI turnover rate was changed before the production of c-MYC protein, it is suggested that PI turnover triggers the production of c-MYC protein, although the molecular mechanism of the devlopmental change in PI turnover is still unknown.

MATERIALS AND METHODS

^{32}P labeling of Phospholipid Wistar rats were anesthetized and their cerebella were dissected. About 50 mg of tissue was homogenized in 400µl reaction buffer (2mM $MgCl_2$, 50mM Tris-HCl pH7.4) with a glass homogenizer. Two sets of 100µl sample solution were used for phosphorylation assay. The samples were preincubated for 5min at 30℃ in the reaction buffer, and incubated at 30℃ for 15 min with 2µCi of [$\gamma^{32}P$]-ATP (approx. 3000Ci/mmol; NEN), as described by Shaikh & Palmer (13). Phospholipids were extracted and applied on a thin layer chromatography (TLC) plate according to the method of Jolles et al. (14). The radioactivity in each lipid was determined by scraping each radioacive spot and scintillation counting, and was normalized with protein concentration.

Immunohistochemistry The strategy for immunohistochemistry was essentially the same as that described by Ito et al (1).

RNA isolation and Northern blot hybridization Total cellular RNA was isolated from the deep frozen cerebellum by the acid phenol method (15). Northern blots were performed by the modified method of Rosen et al. (16). Human c-myc and c-fos probes (Takara, Japan) were labeled using a random primed labeling kit (Boehringer) with [$\alpha^{32}P$]-dCTP (3000mmol/Ci, NEN). Rodent c-jun and jun B synthesized oligomer probes were 5'-end labeled using a Megalabel kit (Takara, Japan) with [$\gamma^{32}P$]-ATP (6000mmol/Ci, NEN). Hybridization was carried out at 42℃ for 24 hr in hybridization solution (50% formamide, 5x SSC, 1x Denhardt's solution, 20mM sodium phosphate, pH 6.5, 0.1% SDS, 0.1mg/ml salmon sperm DNA). The filters were washed to a final stringency of 0.1x SSC, 0.1% SDS at 55℃. Densitometric analysis of X-ray film was performed by an image-analyzer (TIAS-2, ACI, Japan). Each absorption value was normalized with the intensity of ethidium bromide staining of total RNA.

In situ hybridization The c-myc RNA probe was synthesized in vitro. The total RNA from the rat cerebellum was reverse transcribed using moloney murine leukemia virus (M-MLV)

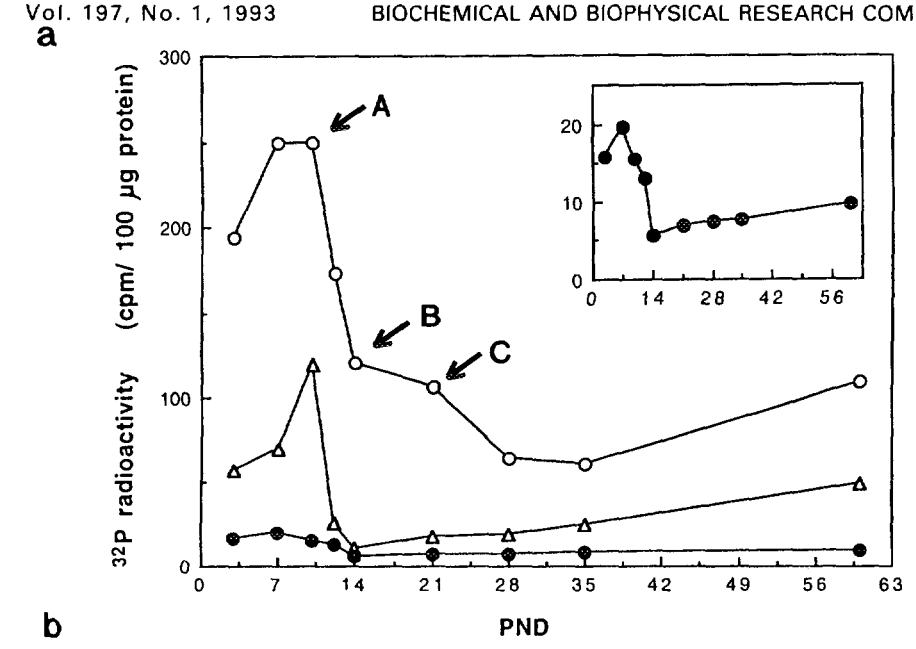
reverse transcriptase (BRL, USA) with random hexamers (Pharmacia, USA). The template DNA fragment for RNA probe synthesis was made by means of the polymerase chain reaction (PCR) . Amplification of the DNA template was performed by touchdown PCR (17). After examining the purity, the PCR product was transcribed to RNA using T7 RNA polymerase (18). Transcription was carried out using a BRL transcription kit (BRL, USA) with digoxigenin-labeling mixture (Boehringer). The resulting RNA fragment corresponded to the antisense strand of c-myc mRNA. On the 50 μm thick cerebellar slice, hybridization was performed overnight at 37℃ in a solution containing 50% formamide, 4xSSC, 1x Denhardt's solution, 0.5 mg/ml salmon sperm DNA, 0.1 mg/ml yeast tRNA, 10 mM DTT, 2mM EDTA, 0.1% SDS, 5%(w/v) dextran sulfate and 1ng/μl digoxigenin-labeled RNA probe. The slices were washed to a final stringency of 0.5xSSC at 37℃. To detect the hybridization signal, digoxigenin-labeled RNA probes were visualized immunohistochemically using anti-digoxigenin peroxidase-conjugated antibody (Boehringer). After washing, peroxidase activity was visualized with diaminobenzidine (Dotaito, Japan) and H_2O_2.

Protein extraction and Immunoblotting About 40 mg of tissue was homogenized with extraction buffer (10mM Tris-HCl, pH 7.4, 1% SDS, 1mM EDTA, 1% 2-mercaptoethanol) at 4℃ with a glass homogenizer and the homogenate was boiled for 2 min. After centrifuging at 10000 xg for 20 min at 20℃, the supernatant was used for immunoblotting as crude protein extract. The nuclear fraction and cytosolic fraction were prepared, as described previously (19). The protein concentration of the extracts was determined by OD_{595} with Coomassie brilliant blue G-250. 20mg of crude extract and cytosolic fraction and 5 mg of nuclear fraction were fractionated onto a 4/20 % gradient SDS-PAGE (20). After electrotransfer (21) of the samples, PVDF membranes were incubated with 1: 50 anti-c-MYC antibody (Cambridge Research Biochemicals, UK). Bound antibody was detected with peroxidase-coupled goat anti-mouse IgG (Bio-Rad). The intensity of the positive band was evaluated using an image-analyzer (TIAS-2, ACI, Japan).

RESULTS

First, we examined ^{32}P-labeling in phospholipids (phosphatidic acid and polyphosphoinositides), because labeling experiments reflect clearly the change in metabolism of the PI cycle. As shown in Fig. 1a, the radioactivity labeled in phosphatidic acid (PA), phosphatidylinositol 4-monophosphate (PIP) and PIP_2 changed remarkably with the number of PND, while the total amount of phospholipid concentration was not changed. The radioactivity of PA, PIP and PIP_2 showed a peak value of around PND 10, then decreased rapidly, reaching the minimum value on PND 14 for PIP and PIP_2, and PND 28 for PA. Then the radioactivity of the labeled phospholipid showed a gradual increase. The parallelism among these three kinds of radio-labeled phospholipids was fairly good.

On PND 10, 14 and 21, we examined the immunohistochemical pattern of rat cerebellar Purkinje cell by anti-PIP_2 Ab. The results obtained in these experiments are shown in Fig. 1b. On PND 10, clear immunoreactivity appeared in dendrites (indicated by white arrow head), nuclei and the perikarya of the Purkinje cells. A weak staining was also observed in the molecular layer (ML), revealing scattered immunopositive cell bodies, which might represent stellate or basket cells. There was no labeling of the external germinal layer (EGL), except for fiber-like staining, where proliferating and premigrational granule cells were present. On the other hand, the



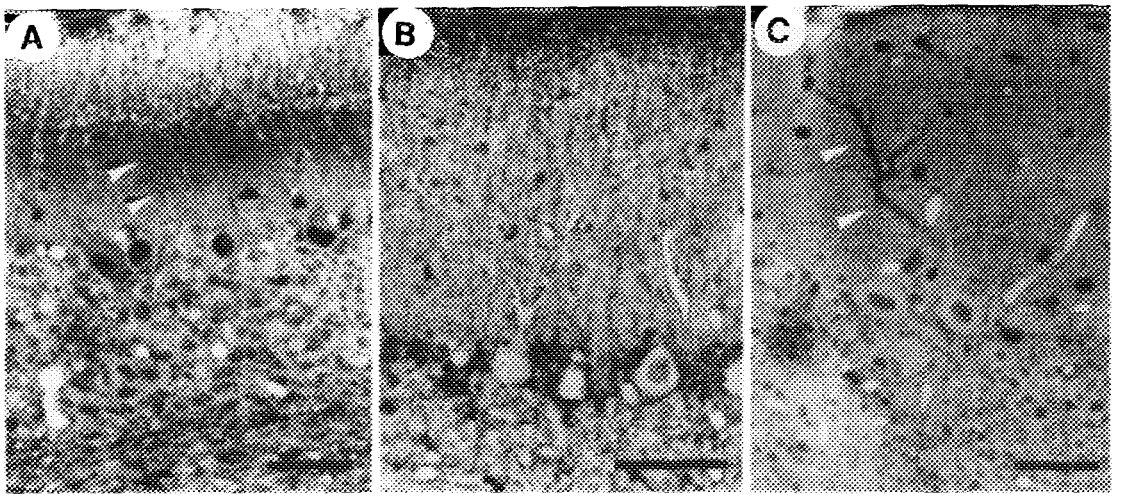


Fig. 1. (a) Developmental changes of the expression of ^{32}P-labeled PA, PIP and PIP2 normalized with protein contents in the rat cerebellum. Changes of PIP2 expression were expanded in the scale in the upper-right column. ○: PA, △: PIP, ●: PIP2
(b) Immunostaining of rat cerebellar slices with anti-PIP2 antibody on PNDs 10 (A), 14 (B) and 21 (C). White arrow head shows densely stained dendritic region of Purkinje cell.
Calibration bar = 50μm.

migratory granule cells in the internal granular layer (IGL) showed immunoreactivity in the nuclei and cytoplasm (Fig. 1b-A). On PND 14, the immunostaining of the Purkinje cell dendrites had almost disappeared. The staining of the perikarya of the Purkinje cells totally disappeared on PND 14, but the reactivity of epithelial glial cells around the soma of the Purkinje cells increased. In ML, fiber-like staining was observed in what seemed to be the Bergmann glial fibers (Fig. 1b-

B). On PND 21, the immunostaining was particularly strong on the distal part of the Purkinje cell dendrites (indicated by the white arrow heads). However, staining was still absent in the perikarya and basal dendritic stems of the Purkinje cells. The immunoreactivity of epithelial glial cells in PND 21 was as weak as that of granule cells (Fig. 1b-C). The difference in the staining pattern between PND 21 and adult (more than PND 35) was quite small (data not shown).

To investigate the correlation between PI turnover and the expression of IEG, we examined the developmental change of the expression of c-myc, c-fos, c-jun and jun B. As shown in Fig.2a, the mRNA level of c-myc showed a peak on PND 7 and then decreased monotonously. On the other hand, c-fos showed a constant level until PND14, and a steep increase after PND 18. The expression of c-jun and jun B showed a similar level change, which resembled that of c-fos. The difference between c-fos and c-jun (=jun B) can be seen after PND 28.

The amount of c-MYC protein was measured by western blotting for cytosol and nucleous, respectively (Fig. 2b). The c-MYC protein in cytosol showed a sharp peak between PND 10 and 12, while nuclear c-MYC protein showed a wide spread around PND 21. Since most c-MYC protein is found to be localized in cytosol (data not shown), MYC protein can play an important role in cytosol.

In order to establish the localization of c-myc, we conducted in situ hybridization experiments for c-myc. As shown in Fig. 3, the stained density reached a maximum on PND 7 and 10, then decreased as the PND increased. On PND 3, the staining was entirely weak. On PND 7, the staining increased enormously throughout the entire area. On PND 10, the upper part of the picture area, the density of the staining faded prominently, while the residual area was still densely stained. When the PND advanced to 14, the upper part, corresponding to Purkinje cell dendrites, were not stained completely. The staining was found to be localized at Purkinje cell soma and several interneurons.

In order to clarify the relationships between PI turnover and MYC protein, we compared the change in the quantities of ^{32}P labeled PIP_2 and c-MYC protein in cytosol. As shown in Fig. 4, ^{32}P incorporation changed initially, and was followed by a change in c-MYC protein. After PND 14, the relationship between the two was reversed.

DISCUSSION

As shown in Fig. 1a and 1b, both immunostaining by anti-PIP_2 Ab and ^{32}P incorporation into polyphosphoinositide was largely reduced until 14 days after birth, after which it recovered gradually. When we examined the developmental change in the expression of IEGs, we found that among several IEGs, only c-myc expression was changed in parallel with PI turnover, as shown in

Fig. 2. As a result of the change in c-myc, c-MYC protein showed a peak on PND 10 and 14 (2 or 3 days later than that of mRNA) and then gradually decreased and eventually disappeared

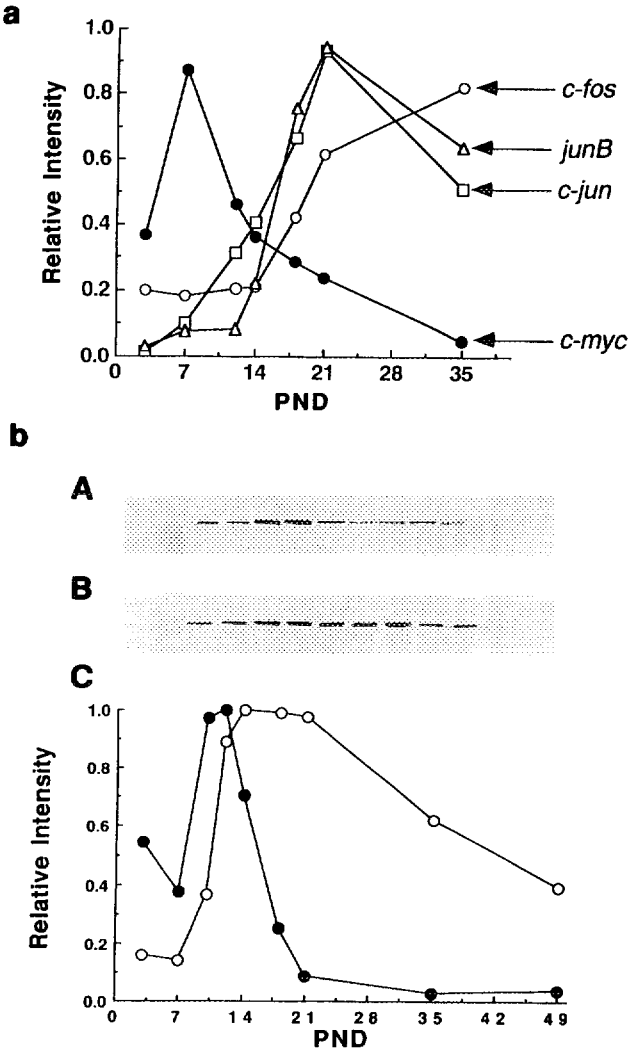


Fig. 2. (a) Developmental changes of the expression of immediate early genes in the rat cerebellum detected with northern blotting. ●: c-myc, ○: c-fos, □: c-jun, △: jun B
We synthesized oligomer probes whose sequences were as follows:
c-jun; 5'-CAA AGC CAG GCG CGC CAC GTC CTT CCC ACT CCA GCA CAT T-3',
jun B; 5'-TAC GCT GGA AGG GGG CCA TGT AAA CTT CGA GGT GGA AGG A-3'.
(b) Developmental changes of c-MYC protein expression in the rat cerebellum detected with Western blotting. (A) c-MYC in cytosolic fraction. (B) c-MYC in nuclear fraction. (C) The developmental change in MYC protein expression in cytosolic fraction (●) and nuclear fraction (○). The amount of cytosol protein was about 6 times larger than that of nucleus.

after PND 28. These developmental changes in the quantities of c-myc gene and c-MYC protein were in approximate agreement with previously reported data (3, 4). In order to make clear the cause-response relationships between PI turnover and c-MYC protein, we compared both of them developmentally, as shown in Fig.4. The incorporation of ^{32}P into PIP2 changed in parallel

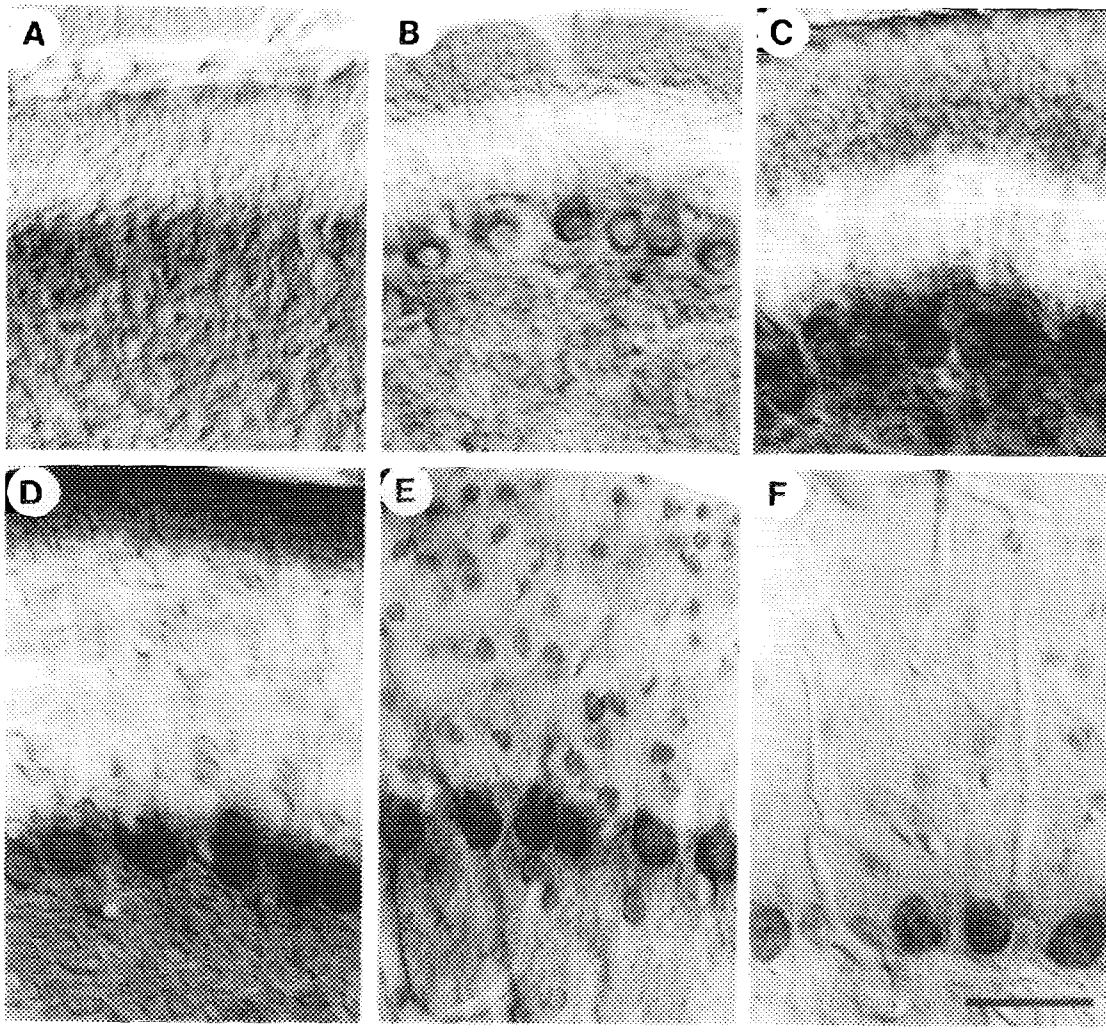


Fig. 3. In situ hybridization of digoxigenin-labeled c-myc RNA probe in the rat cerebellum on PND 3 (A), 7 (B), 10 (C), 14 (D), 21 (E) and adult (F). Calibration bar = 50μm.
We used PCR primers whose sequences were as follows:
CM-1 (5'-primer) 5'-GAC AGG ACT CCC CAG GCG CAG-3'
CM-2 (3'-primer) 5'-(TAATACGACTCACTATA)"GGGA" GCT CAC GTT GAG GGG CAT CG-3'
Primer CM2 had the T7 RNA polymerase promoter sequence (in parentheses) and transcription initiating sequence (in quotation marks) attached to its 5' end. This primer set amplified 172bp of rat c-myc protooncogene cDNA nested exon 1-exon 2 junction.

with c-myc. Accordingly, the PI turnover rate was changed before the expression of c-MYC protein was changed.

The following two hypotheses are likely to explain the results: One possible explanation is to assume an unknown regulating factor which controls both c-myc expression and PI turnover rate, simultaneously. However, because of limited information, it is unreasonable to assume such a

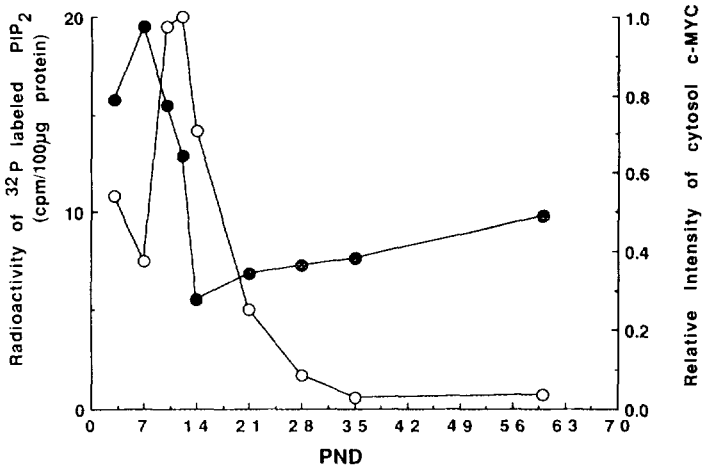


Fig. 4. Correlation between ^{32}P-labeled PIP2 and cytosolic c-MYC protein expression. ●: ^{32}P-labeled PIP2, ○: c-MYC protein expression in cytosol.

signaling mechanism. Another more reasonable explanation is to assume that the hydrolysis of PIP2 stimulates c-myc expression. According to the results shown in Fig. 1b, density changes in PIP2 staining are ascribed to Purkinje cells, not to granule cells. If PIP2 staining density reflects PI turnover rate, the hypothesis described above may hold in Purkinje cells.

It seemed at first sight strange that the immunostaining and ^{32}P incorporation changed in parallel with the number of PND, because ^{32}P incorporation does not reflect the total amount of PIP2. Immunostaining for protein molecules, as far as is known, might be evidence that the protein molecule actually exists. In the case of the immunostaining of the phospholipid molecule, however, the stained density will express the degree of interaction between phospholipids and protein, because phospholipid molecules could not be fixed by ordinary fixation procedures (1). Therefore, the staining pattern shown in Fig. 1b may depend on the molecular distance between PIP2 and PIP2 binding protein, such as PIP2 specific phospholipase C (PLC) or PIP2 phosphatase. In this case, the region stained by anti-PIP2 Ab shows that the distance between PIP2 and PLC may be close. If that is the case, IP3 induced Ca release may appear only in the stained part of the tissue. This has already been reported by us using the same preparation (1). Localization of the immunostained region was examined using anti-PIP2 Ab and was compared to that of c-myc expression by the method of in situ hybridization, developmentally. During the cerebellar development, the c-myc gene was expressed densely at Purkinje cell soma, showed a peak on PND 14 and gradually reduced. Although the staining of granule cells appeared on PND 10 and 14, the major contribution of c-myc gene expression in the tissue in Fig. 2 might be due to Purkinje cells. Immunostaining by anti-PIP2 Ab was also found to be concentrated in Purkinje cells. Therefore, the correlation between PI turnover and c-myc may hold mainly in Purkinje cells.

In conclusion, PI turnover may regulate c-myc expression in rat cerebellar Purkinje cell during development until PND 14.

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