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DEVELOPMENTAL CHANGES IN THE ACTIVITIES OF PHOSPHOLIPASE C, 3-KINASE, AND 5-PHOSPHATASE IN RAT BRAIN

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SUMMARY: The specific activities of phospholipase C, 3-kinase, and 5-phosphatase were measured in brain homogenates from rats at different developmental stages. The activities of 3-kinase and 5-phosphatase increased by 14-fold and 2-fold, respectively, during development from fetus to adult, while PLC activity remained constant. These results suggest that the metabolism of inositol phosphates varies widely during development. In young brain stimulated by an agonist, it is predictable that Ins(1,4,5)P_3 lasts longer and its average concentration is higher than in adult brain. The opposite is true for both the lifetime and concentration of Ins(1,3,4,5)P_4. These developmental changes will invariably affect the property of Ca^{2+} oscillation and the effective time during which cells respond to the Ca^{2+}-mobilizing agonists.

Ins(1,4,5)P_3, a well-established second messenger generated by phospholipase C (PLC) in response to a variety of extracellular signals, causes the release of Ca^{2+} from internal stores before being rapidly metabolized (1). Two separate routes are known for the elimination of Ins(1,4,5)P_3: Dephosphorylation to Ins(1,4)P_2 by 5-phosphatase (2-4) and 3-kinase-dependent phosphorylation to Ins(1,3,4,5)P_4 (5-7), followed by dephosphorylation to Ins(1,3,4)P_3 by the same 5-phosphatase which converts Ins(1,4,5)P_3 to Ins(1,4)P_2 (8-10). It is generally believed that Ins(1,4)P_2 and Ins(1,3,4)P_3 are degradation intermediates of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, respectively, with no known biological function, while Ins(1,3,4,5)P_4 has been suggested as a second messenger controlling the size of the Ca^{2+}-pool responding to Ins(1,4,5)P_3 (11,12). Thus, alteration in the activities of the three Ins(1,4,5)P_3-related enzymes (PLC, 3-kinase, and 5-phosphatase) would result in changes

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Abbreviations Used: Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate; Ins(1,4)P_2, inositol 1,4-bisphosphate; Ins(1,3,4)P_3, inositol 1,3,4-trisphosphate; PLC, phosphoinositide-specific phospholipase C; 3-kinase, a kinase which converts Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4; 5-phosphatase, inositol polyphosphate-5-phosphatase.

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in the intracellular concentrations and lifetimes of the two second messengers, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. In this study we measured the activities of the Ins(1,4,5)P_3-related enzymes with the aim of possibly finding that the flux of inositol polyphosphates varies during ontogeny.

MATERIALS AND METHODS

Materials. [^{3}H]Ins(1,4,5)P_3, [^{3}H]Ins(1,4)P_2, and [^{3}H]Ins(1,3,4,5)P_4 were obtained from New England Nuclear. The Partisil SAX HPLC column was purchased from Whatman. Dowex AG1-X2 (formate) was purchased from Bio-Rad. Sprague-Dawley rats were obtained from Taconic Farm (Germantown, NY).

Homogenate Preparation. Brains were rapidly removed from Sprague-Dawley rats at different developmental stages after decapitation, rinsed with 0.15 M NaCl, and frozen with liquid nitrogen. Homogenization buffer (50 mM Tris/HCl, pH 7.5, 250 mM sucrose, 3 mM $MgCl_2$, 1.5 mM EGTA, 1 mM DTT, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 mM PMSF) was added to the frozen brains at 5 ml per gram of tissue. The mixture was homogenized with a Brinkman Polytron (3 times, 20 sec each at scale 7). The homogenate was centrifuged at 1000 x g for 15 min to remove nuclei and tissue debris. The resulting supernatant was used as a crude source of phospholipase C, 5-phosphatase, and 3-kinase. Protein concentration was estimated by using the modified Lowry reagent (BCA reagent by Pierce Chemical Co.) according to the manufacturer's procedure.

Phospholipase C Assay. The formation of water soluble [^{3}H]inositol phosphate from [^{3}H]phosphatidylinositol was followed at pH 7.0 in the presence of 0.1% deoxycholate, 3 mM $MgCl_2$, and 1 mM EGTA as described previously (13).

3-Kinase Assay. IP_3-kinase activity was measured by the production of $InsP_4$ from Ins(1,4,5)P_3. The incubation mixture (0.2 ml) contained 50 mM Tris/HCl, pH 7.5, 100 mM KCl, 10 mM ATP, 20 mM $MgCl_2$, 1 mM DTT, 5 mM 2,3-diphosphoglycerate, 2 mM EGTA, 2 μM Ins(1,4,5)P_3, and 20,000 cpm of [^{3}H]Ins(1,4,5)P_3. The reaction was initiated by adding the crude enzyme (5-20 μl), followed by incubation for 2 min at 37°C. The reaction was terminated by adding 50 μl of 1 N H_3PO_4, followed by 50 μl 1 N NaOH. The inositol phosphates were separated on a 0.7 ml Dowex AG 1-X2 (formate form) column. Each column was washed with 12 ml of 0.5 M ammonium formate containing 0.1 M formic acid to remove unreacted Ins(1,4,5)P_3. The ammonium concentration was increased to 1.5 M for elution of Ins(1,3,4,5)P_4 in 1.5 ml. The identity of the product, $InsP_4$, was confirmed by its identical retention time with the authentic [^{3}H]Ins(1,3,4,5)P_4 on analysis on a Partisil SAX10 HPLC column eluted as described (14).

5-Phosphatase Assay. Dephosphorylated product of Ins(1,4,5)P_3 was measured. The incubation mixture (0.2 ml) contained 50 mM Hepes/KOH, pH 7.0, 1 mM $MgCl_2$, 1 mM DTT, 30 μM Ins(1,4,5)P_3, and 20,000 cpm of [^{3}H]Ins(1,4,5)P_3. The reaction was initiated by adding the crude enzyme (10-20 μl), followed by incubation for 2 min at 37°C. Termination of the reaction as well as separation of inositol phosphates was achieved as described for the 3-kinase assay, except that $InsP_2$ was eluted with 4 ml of 0.4 M ammonium formate in 0.1 M formic acid. More than 95% of the product was Ins(1,4)P_2 when analyzed on a Partisil SAX10 HPLC column.

RESULTS AND DISCUSSION

Figure 1 shows developmental changes in the specific activities of Ins(1,4,5)P_3-related enzymes--PLC, 3-kinase, and 5-phosphatase. The 3-kinase activity is

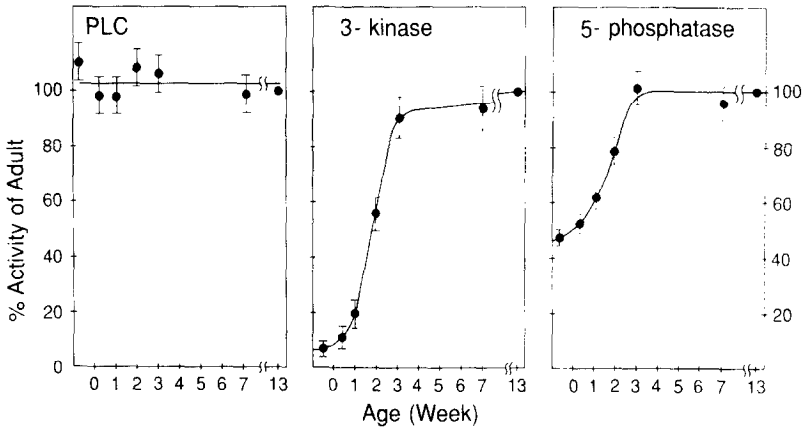

Figure 1. Developmental changes of the specific activities of phospholipase C, 3-kinase, and 5-phosphatase in rat brain. Enzyme activities are measured in brain homogenates from rats at different ages (from 4 days before birth to 13 weeks of age) and expressed as the percentage of 13-week-old rat in which the specific activities of PLC, 3-kinase, and 5-phosphatase are 19, 1.1., and 25 nmole/min per mg of protein, respectively, under the assay conditions described in Materials and Methods. The results (mean ±SD) are means of three separate experiments.

very low in the fetus and within 1 week of age, increases rapidly between 1 and 3 weeks, and further increases slowly up to 13 weeks of age. The 5-phosphatase activity in the fetus was about 50% of maximal activity, increased rapidly until 3 weeks, and remained constant between 3 and 13 weeks. During the entire developmental stage, the PLC activity remained constant. More than 95% of the dephosphorylated product of Ins(1,4,5)P_3 by brain homogenates was Ins(1,4)P_2, suggesting that the dephosphorylation reaction was specific to the 5-PO_4 of inositol. However, this result does not eliminate the possibility that the 2-fold increase in 5-phosphatase activity is partly due to increase in non-specific phosphatase (e.g., alkaline phosphatase) which might prefer 5-PO_4 over 1- and 4-PO_4 of inositol. In the case of the 3-kinase assay, the enormous increase (14-fold from the fetus to the adult) and the confirmation of the product as Ins(1,3,4,5)P_4 preclude any explanation except that the apparent increase is due to an elevation in the activity of 3-kinase specific to inositol phosphate. The increase in the activity of 3-kinase and 5-phosphatase does not appear to be due to the variation in the concentration of the substance which activates or inhibits the enzymes. A mixing experiment in which an equal mixture of fetus and adult homogenates is assayed for either 3-kinase or 5-phosphatase yielded a value, which is expected to be within experimental error, from two separate measurements of the homogenates (data not shown). It is therefore likely that the activity increases reflect true increases in enzyme concentration. However, a final ruling should wait until antibodies to 3-kinase and 5-phosphatase become available.

Enzyme activity is often regulated by covalent modification such as protein phosphorylation. Among four 5-phosphatases purified to date (45 and 75 KDa enzymes from human platelets (8,10), and 66 and 160 KDa enzymes from rat brain (9)), the 45 (15) and 66 KDa (9) enzymes are known to be activated by protein kinase C-dependent phosphorylation. The 53 KDa 3-kinase purified from rat brain (16) also appears to be regulated by both protein kinase C (17) and calmodulin-dependent kinase (S.S. Sim, J.W. Kim, and S.G. Rhee, unpublished results). Therefore, it is also a possibility that the increase in the activities of 3-kinase and 5-phosphatase during ontogeny is due to the change in the state of covalent modification. Previous reports showed that 5-phosphatase (18) in rat brain can be divided in a 3 to 7 ratio between the 180,000 x g supernatant and pellet, while most of the 3-kinase (16) is found in cytosol. We also measured their distribution in rat brain. No significant trend was observed: ~60% of 5-phosphatase and ~20% of 3-kinase were found in the 180,000 x g pellet irrespective of developmental stage (data not shown).

Since the initial observation of receptor-linked breakdown of phosphoinositide, the role of Ins(1,4,5)P_3 as a messenger mobilizing Ca^{2+} has been firmly established (11,12,19). Recently, it is also emerging that in most tissues the intracellular Ca^{2+} regulation is not mediated by Ins(1,4,5)P_3 alone, but rather by a duet between Ins(1,4,5)P_3 and its essential partner, Ins(1,3,4,5)P_4 (12). Ins(1,3,4,5)P_3 is believed to control the formation of the Ca^{2+}- carrying junction between two distinct pools of Ca^{2+}, one which is sensitive to Ins(1,4,5)P_3 and the other which is insensitive to Ins(1,4,5)P_3. Despite the fact that almost all animal cells maintain a specific 3-kinase and go to the trouble of making Ins(1,3,4,5)P_4 by consuming an ATP molecule, the physiological role of Ins(1,3,4,5)P_4 has been difficult to unravel and is still under dispute. Part of the reason is that the demonstration of synergism between Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 probably requires a low level of 3-kinase activity in cells. It is known that both the 3-kinase activity and the concentration of Ins(1,3,4,5)P_4 change widely between tissues and cells.

Our current study demonstrates that the levels of 5-phosphatase and 3-kinase increase during development, while PLC activity remains unchanged. This means that only the capacity to metabolize, but not the capacity to generate, Ins(1,4,5)P_3 increases. Consequently, it is expected that the lifetime of the Ins(1,4,5)P_3 molecule becomes shorter and the average concentration of Ins(1,4,5)P_3 during stimulation becomes lower as the brain develops. In addition, from the fetus to adult, the 3-kinase increases as much as 14-fold while 5-phosphatase only increases 2-fold. Since the same 5-phosphatase which dephosphorylates Ins(1,4,5)P_3 also degrades Ins(1,3,4,5)P_4, the capacity to generate Ins(1,3,4,5)P_4 increases much faster than the capacity to degrade it as the brain develops. This will enhance the average concentration of Ins(1,3,4,5)P_4 during stimulation, in contrast to the decreased concentration of Ins(1,4,5)P_3 in the adult brain. One obvious consequence

will be the changes in the property of Ca^{2+} oscillation (20) including the height of the spike and the frequency of oscillation. Since the levels of both the 3-kinase and the 5-phosphatase are lower in the younger brain, Ins(1,4,5)P_3 and the product of its phosphorylation, Ins(1,3,4,5)P_4, are present to function as second messengers for longer periods of time than in the adult brain. This might be related to the fact that the same two second messengers are used for both long-term (e.g., growth) and short-term (e.g., secretion) responses and that the younger brain may require longer-term responses to agonists for normal growth and development. Whether all of these predictable phenomena actually occur during development remains to be shown.

REFERENCES

1. Berridge, M.J., and Irvine, R.F. (1984) Nature (London) 312, 315–321.
2. Downes, C.P., Mussat, M.C., and Michell, R.H. (1982) Biochem. J. 203, 169–177.
3. Majerus, P.W., Connolly, T.M., Deckmyn, H., Ross, T.S., Bross, T.E., Ishii, H., Bansal, V.S., and Wilson, D.B. (1986) Science 234, 1519–1526.
4. Connolly, T.M., Bansal, V.S., Bross, T.E., Irvine, R.F., and Majerus, P.W. (1987) J. Biol. Chem. 262, 2146–2149.
5. Irvine, R.F., Letcher, A.J., Heslop, J.P., and Berridge, M.J. (1986) Nature (London) 320, 631–634.
6. Biden, T.J., Comte, M., Cox, J.A., and Wollheim, C.B. (1987) J. Biol. Chem. 262, 9437-9440.
7. Ryu, S.H., Lee, S.Y., Lee, K.Y., and Rhee, S.G. (1987) FASEB J. 1, 388–393.
8. Connolly, T.M., Bross, T.E., and Majerus, P.W. (1985) J. Biol. Chem. 260, 7868–7874.
9. Hansen, C.A., Johanson, R.A., Williamson, M.T., and Williamson, J.R. (1987) J. Biol. Chem. 262, 17319–17326.
10. Mitchell, C.A., Connolly, T.M., and Majerus, P.W. (1989) J. Biol. Chem. 264, 8873–8877.
11. Irvine, R.F., and Moor, R.M. (1986) Biochem. J. 240, 917–920.
12. Irvine, R.F., Moor, R.M., Pollock, W.K., Smith, P.M., and Wreggett, K.A. (1988) Phil Trans. R. Soc. London B320, 281–298.
13. Ryu, S.H., Cho, K.S., Lee, K.Y., Suh, P.G., and Rhee, S.G. (1987) Biochem. Biophys. Res. Commun. 141, 137–144.
14. Dean, N.M., and Moyer, J.D. (1987) Biochem. J. 242, 361–366.
15. Connolly, T.M., Lawing, W.J., Jr., and Majerus, P.W. (1986) Cell 46, 951–958.
16. Johanson, R.A., Hansen, C.A., and Williamson, J.R. (1988) J. Biol. Chem. 263, 7465–7471.
17. Imboden, J.B., and Pattison, G. (1987) J. Clin. Invest. 79, 1538-1541.
18. Erneux, C., Delvaux, A., Moreau, C., and Dumont, J.E. (1986) Biochem. Biophys. Res. Commun. 134, 351–358.
19. Putney, J.W., Jr. (1986) Cell Calcium 7, 1–2.
20. Berridge, M.J., Cobbold, P.H., and Cuthbertson, K.S.R. (1988) Phil. Trans. Roy. Soc. Lond. B320, 325–334.