Cytidine–Diphosphate Diacylglycerol Labeling as an Index of Inositol Lipid-Mediated Signal Transduction in Brain and Neural Cells

Anne M. Heacock, Evan B. Stubbs, Jr., and Bernard W. Agranoff

Neuroscience Lab, University of Michigan, Ann Arbor, Michigan 48104-1687

A method for assessing stimulated phosphoinositide turnover by measurement of the liponucleotide CDP-diacylglycerol is presented. The phosphoinositide signal transduction pathway consists of a sequence of reactions in which the second messengers inositol 1,4,5-triphosphate and diacylglycerol are recycled back to phosphatidylinositol (PtdIns), which then serves to replenish the initial hydrolyzed substrate, phosphatidylinositol 4,5-bisphosphate. Receptor-stimulated inositol lipid turnover is most commonly assessed by measurement of the accumulation of [^{3}H]inositol-labeled inositol phosphates in the presence of Li^+. The latter blocks inositol monophosphatase and thus can lead to a depletion of intracellular inositol. Because inositol is required for resynthesis of PtdIns, the immediate precursor of PtdIns, CDP-diacylglycerol, also accumulates in the presence of agonist and Li^+. Measurement of radiolabeling of this liponucleotide following incorporation of [^{3}H]cytidine thus forms the basis for an alternative assay for inositol lipid turnover. The general applicability of this method may be limited, since, in brain slices, not all receptors exhibit CDP-diacylglycerol responses that are consistent with their inositol phosphate responses. In addition, in cultured neural cells, growth in inositol-free, chemically defined medium is required to maximize the Li^+-dependent CDP-diacylglycerol response. A major advantage of this method may be its ability to provide insight into the regulation of phosphoinositide turnover since this method uniquely reflects slowing of the regenerative cycle. Such *in vitro* studies may have relevance to the *in vivo* action of Li^+ as a psychotherapeutic agent. © 1993 Academic Press, Inc.

The phosphoinositide signal transduction pathway in the CNS can be activated by any number of receptors that may be differentially distributed within the brain (1). Binding of agonists to these receptors results in a G-protein-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$) to yield equimolar amounts of inositol 1,4,5-trisphosphate (InsP$_3$) and diacylglycerol (DG) (Fig. 1). Although methods for measuring the chemical mass of these two second messengers are available

able (2, 3), phosphoinositide turnover is more commonly assessed by determination of radiolabeled metabolites of either or both of these compounds. This signal transduction pathway was originally assayed via measurement of the incorporation of $^{32}P_i$ into phosphatidic acid and phosphatidylinositol (4), intermediates in the compensatory resynthesis of polyphosphoinositides (Fig. 1). This method was subsequently supplanted by one based on the ability of Li^+ to uncompetitively inhibit inositol monophosphatase (5) and thus "trap" [^{3}H]inositol-labeled metabolites of InsP$_3$ as inositol monophosphate (6). This action of Li^+, together with the high K_m for inositol exhibited by the biosynthetic enzyme phosphatidylinositol synthase (7, 8), predicted that during receptor activation the presence of Li^+ would disrupt the cycle depicted in Fig. 1 by causing a reduction in intracellular inositol which would lead to a diminished resynthesis of phosphatidylinositol (6, 9). Support for this model of Li^+ action has recently been obtained in experiments with carbachol-stimulated rat cerebral cortex slices in which, following labeling with [^{3}H]cytidine, the precursor of phosphatidylinositol (and of the mitochondrial phosphatidylglycerols), CDP-diacylglycerol (CMP-phosphatidic acid, CMP-PtdOH), was found to accumulate in a Li^+-dependent manner (10, 11). This metabolite of diacylglycerol is indirectly "trapped" by Li^+ since its further metabolism to phosphatidylinositol is compromised by the Li^+-induced decrease in intracellular inositol. Assessment of phosphoinositide turnover by measurement of [^{3}H]CDP-diacylglycerol accumulation has the advantage that it is easier and quicker than other available assays and it yields information regarding the metabolic interrelationships within this signal transduction pathway. However, there are some limitations to the applicability of this method, as detailed below.

METHOD

Tissue Preparation

Brain Slices

Rat brains were rapidly removed following decapitation, placed into ice-cold saline, and dissected into the desired

regions. Tissue slices (350 × 350 μm) were prepared as described previously (12) on a McIlwain tissue chopper and then washed three or four times at 37°C in oxygenated buffer A (142 mM NaCl, 5.6 mM, KCl, 2.2 mM CaCl$_2$, 3.6 mM NaHCO$_3$, 1 mM MgCl$_2$, 5.6 mM glucose, and 30 mM Hepes, pH 7.4). Slices were then incubated in a flask at 37°C, with agitation in 50 ml of the same buffer for 60 min and one change of buffer after 30 min.

Cultured Cells

Human SK-N-SH neuroblastoma cells were cultured either in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum or in an inositol-free chemically defined (IFCD) growth medium (13). The latter was changed daily for 5–7 days in order to deplete intracellular inositol. Cells grown to confluency (7–10 days) were harvested with Puck's D$_1$ solution, washed once with buffer A, and then resuspended at a concentration of 1–2 mg of protein/ml.

[^{3}H]CDP-Diacylglycerol Assay

Gravity-packed slices (50 μl; 0.8–1.2 mg protein) or cell suspension (~0.3 mg protein) were transferred to flat-bottom polypropylene minivials and incubated in a final volume of 500 μl with buffer A containing 3 μCi of [5-^{3}H]cytidine (15–25 Ci/mmol; Sigma Chemical Co., St. Louis, MO) at 37°C for 60 min. Evaluation of other

sources of [^{3}H]cytidine indicated that the Sigma product had the lowest amount of interfering, organic solvent-extractable contaminants, less than 2% of the basal chloroform-extractable radioactivity. After the 60 min prelabeling period, buffer A with or without LiCl (final concentration 5 or 10 mM, as indicated) was added, followed after 10 min by either agonist or vehicle (buffer A). After 30 min, reactions were stopped by addition of 1.7 ml of chloroform:methanol (1:2, vol/vol). Phases were then separated by addition of 1 ml of chloroform and 0.5 ml of 1.2 M HCl. Following low-speed centrifugation, the lower organic phase was transferred to a clean tube and washed twice with 2 ml of acidified Folch theoretical upper phase (chloroform:methanol:H$_2$O, 3:47:48 by vol, with 0.5 ml concentrated HCl added per 60 ml). A 1-ml aliquot of the washed organic phase was then transferred to a scintillation vial and dried prior to scintillation counting. Identity of the organic-extractable radiolabeled product with CDP-diacylglycerol was verified by thin-layer chromatography on oxalate-impregnated plates with the solvent chloroform:acetone:methanol:acetic acid:water (40: 15:13:12:8, by vol) (14). Inositol concentration was determined by the method of Mount and Laker (15).

Use with Brain Slices

Examination of the ability of several phosphoinositide-linked receptors to elicit a CDP-diacylglycerol response revealed striking differences among them (16) (Table 1). In both cortex and hippocampus, activation of the mus-
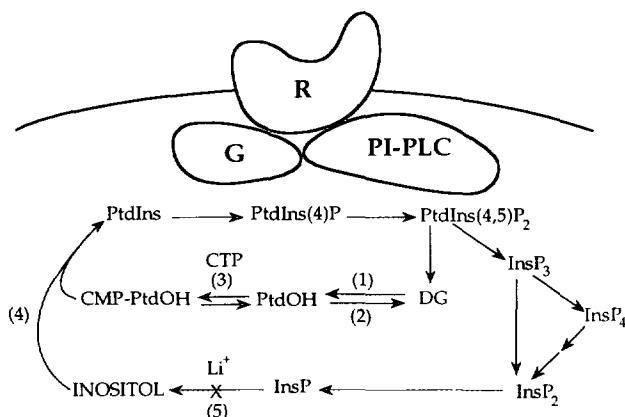

FIG. 1. Phosphoinositide signal transduction pathway. Binding of an agonist to its receptor (R) results in activation of a guanine nucleotide binding protein (G), which then interacts with phosphoinositidase C (PI-PLC) to enhance hydrolysis of PtdIns(4,5)P$_2$ to InsP$_3$ and DG. InsP$_3$ is metabolized to InsP (phosphate in the 1, 3, or 4 position), which is dephosphorylated by inositol monophosphatase (5) to inositol in a Li$^+$-sensitive manner. DG may be acted on by diglyceride kinase (1) to yield phosphatidic acid (PtdOH), which may be either dephosphorylated to give DG, a reaction catalyzed by phosphatidate phosphohydrolase (2), or combined with CTP, via CTP:phosphatidate cytidyltransferase (3), which catalyzes a reversible reaction, to give CDP-diacylglycerol (CMP-PtdOH), a substrate for phosphatidylinositol synthase (4). Additional routes for metabolism of CDP-diacylglycerol (not shown) include cleavage via CDP-diacylglycerol hydrolase, a lysosomal enzyme, or utilization for synthesis of phosphatidylglycerols in mitochondria.

TABLE 1

Comparison of Inositol Phosphate and CDP-Diacylglycerol Responses in Brain Slices

	Percentage stimulation above basal	
	InsP	CMP-PtdOH
Cortex		
Carbachol (1 mM)	793 ± 156	973 ± 182
Quisqualate (100 μM)	611 ± 150	130 ± 41
Endothelin (0.5 μM)	613 ± 30	10 ± 6
Hippocampus		
Carbachol (1 mM)	807 ± 141	1056 ± 136
Quisqualate (100 μM)	1514 ± 243	310 ± 50
Endothelin (0.5 μM)	1062 ± 139	12 ± 5

Note. Slices of cortex or hippocampus from 13 to 18-day-old rats were prelabeled with [^{3}H]inositol or [^{3}H]cytidine and then incubated with agonists for 30 min in the presence of 5 mM LiCl. Radioactivity in inositol phosphates (InsP) or CDP-diacylglycerol (CMP-PtdOH) was then determined. Data (taken from Ref. 16, Fig. 1) are expressed as percentage stimulation above basal (mean ± SEM, n = 4–6). All of the CDP-diacylglycerol response and ~80% of the inositol phosphate response were dependent on the presence of Li$^+$. Inclusion of 10–30 mM inositol prevented the accumulation of CDP-diacylglycerol (16).

carinic receptor with carbachol resulted in stimulations of CDP-diacylglycerol accumulation comparable to those observed for inositol phosphates. For the metabotropic glutamate receptor agonist quisqualate, these responses were less well correlated. For endothelin, which gave a robust inositol phosphate response, the CDP-diacylglycerol response was barely detectable. This result underlines the inappropriateness of the CDP-diacylglycerol method as a primary screen for phosphoinositide-linked receptors but suggests that the use of both assays may provide additional insight into the regulation of this signal transduction pathway. A one-to-one relationship between these two assay methods would not necessarily be expected, since neither response accurately reflects chemical mass. The lack of a response to endothelin may relate to the reversibility of the CDP-diacylglycerol biosynthetic reaction, which would allow the possibility of loss of CDP-diacylglycerol to phosphatidic acid and then to diacylglycerol. Alternatively, there may be a higher inositol content in the vicinity of the endothelin receptors, which would permit the CDP-diacylglycerol to escape the Li^+ block and be metabolized to phosphatidylinositol. Intact rat brain has a relatively high inositol content (5–6 mM) (17) but this is reduced by up to 80% upon incubation of brain slices (16, 17). That the resulting low inositol concentrations of 1 mM or less are crucial to the ability to observe Li^+-dependent agonist-stimulated CDP-diacylglycerol accumulation is indicated by experiments with cultured neural cells.

Use with Cultured Cells

SK-N-SH neuroblastoma cells grown in standard culture medium showed a fivefold increase in [^{3}H]CDP-diacylglycerol labeling in response to muscarinic receptor activation (Table 2) (18). In contrast to the results with rat brain slices, this response was insensitive to Li^+ and was not reversible by addition of 10 mM inositol. Measurement of the intracellular inositol content indicated a concentration of 6 mM (18), well above the K_m for phosphatidylinositol synthase (0.9 mM) observed in these cells. Growth in inositol-free chemically defined medium for 5–7 days resulted in a drop in inositol to $\leq$0.5 mM. Cells grown under these conditions exhibited a robust Li^+-dependent accumulation of [^{3}H]CDP-diacylglycerol in the presence of carbachol (Table 2), which could be substantially reversed by the addition of 10 mM inositol.

CONCLUDING REMARKS

Measurement of [^{3}H]CDP-diacylglycerol is a convenient index of stimulated phosphoinositide turnover that is somewhat less time-consuming to carry out than the inositol phosphate assay. In addition to its use in brain slices and neuroblastoma cells, the CDP-diacylglycerol assay has been applied to parotid slices (14), neurotrophils (19), platelets (20), and ileum (21). It also forms the basis for an autoradiographic method for localization of receptor-mediated phosphoinositide turnover in brain slices (22). The limitations of this assay method arise from the indirect nature of the Li^+ "trapping" of CDP-diacylglycerol and the requirement for relatively low resting intracellular inositol concentrations. Its use in conjunction with the inositol phosphate assay may further our understanding of the metabolic interrelationships within this signal transduction pathway, especially in regard to inositol homeostasis and the effects of Li^+. The agonist-stimulated, Li^+-dependent accumulation of CDP-diacylglycerol observed *in vitro* gives support to an inositol depletion hypothesis for the psychotherapeutic actions of Li^+ (6, 9). This hypothesis [which is also discussed elsewhere in this issue by Challis *et al.* (23)] predicts that the inhibition of inositol monophosphatase by Li^+ administration *in vivo* results in a drop in intracellular inositol in the brain, a consequent reduction in the resynthesis of phosphoinositide, and diminished second-messenger production. It should be noted that while small reductions in rat brain inositol content have been observed following Li^+ treatment *in vivo* (17), these would appear to be insufficient to compromise the function of the phosphoinositide signal transduction pathway. Thus, the validity of this hypothesis remains to be proven.

TABLE 2

CDP-Diacylglycerol Response to Carbachol
in SK-N-SH Neuroblastoma Cells

	Culture medium			
	Standard		IFCD	
LiCl (10 mM)	Basal	Carbachol	Basal	Carbachol
−	314 ± 47	1624 ± 82	315 ± 32	874 ± 49
+	307 ± 60	1533 ± 45	335 ± 44	7523 ± 1108

Note. [^{3}H]Cytidine-prelabeled cells (0.3 mg protein) grown in either standard or IFCD medium were treated with either buffer or LiCl for 10 min, followed by incubation in the presence or absence of carbachol (1 or 10 mM) for 30 min. Data shown are dpm in [^{3}H]CDP-diacylglycerol, means ± SEM (n = 3; Ref. 18).

REFERENCES

1. Fisher, S. K., Heacock, A. M., and Agranoff, B. W. (1992) *J. Neurochem.* **58,** 18–38.

2. Palmer, S., Hughes, K. T., Lee, D. Y., and Wakelam, M. J. O. (1989) *Cell. Signalling* **1,** 147–156.

3. Preiss, J., Loomis, C. R., Bishop, W. R., Stein, R., Niedel, J. E., and Bell, R. M. (1986) *J. Biol. Chem.* **261,** 8597–8600.

4. Hokin, L. E., and Hokin, M. R. (1955) *Biochim. Biophys. Acta* **18**, 102-110.

5. Allison, J. H., and Stewart, M. A. (1971) *Nature* **233**, 267-268.

6. Berridge, M. J., Downes, C. P., and Hanley, M. R. (1982) *Biochem. J.* **206**, 587-595.

7. Benjamins, J. A., and Agranoff, B. W. (1969) *J. Neurochem.* **16**, 513-527.

8. Ghalayini, A., and Eichberg, J. (1985) *J. Neurochem.* **44**, 175-182.

9. Berridge, M. J., Downes, C. P., and Hanley, M. R. (1989) *Cell* **59**, 411-419.

10. Godfrey, P. P. (1989) *Biochem. J.* **258**, 621-624.

11. Kennedy, E. D., Challiss, R. A. J., Ragan, C. I., and Nahorski, S. R. (1990) *Biochem. J.* **267**, 781-786.

12. Heacock, A. M., Fisher, S. K., and Agranoff, B. W. (1987) *J. Neurochem.* **48**, 1904-1911.

13. Bottenstein, J., and Sato, G. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 514-517.

14. Downes, C. P., and Stone, A. (1986) *Biochem. J.* **234**, 199-204.

15. Mount, J. N., and Laker, M. F. (1981) *J. Chromatogr.* **226**, 191-197.

16. Heacock, A. M., Seguin, E. B., and Agranoff, B. W. (1993) *J. Neurochem.* **60**, 1087-1092.

17. Sherman, W. R., Gish, B. G., Honchar, M. P., and Munsell, L. Y. (1986) *Fed. Proc.* **45**, 2639-2646.

18. Stubbs, E. B., Jr., and Agranoff, B. W. (1993) *J. Neurochem.* **60**, 1292-1299.

19. Stubbs, E. B., Jr., Walker, B. A. M., Owens, C. A., Ward, P. A., and Agranoff, B. W. (1992) *J. Immunol.* **148**, 2242-2247.

20. Watson, S. P., Shipman, L., and Godfrey, P. P. (1990) *Eur. J. Pharmacol.* (Mol. Pharmacol. Section) **188**, 273-276.

21. Godfrey, P. P., and Watson, S. P. (1990) *Dupont Biotech Update* **5**, 74-75.

22. Hwang, P. M., Bredt, D. S., and Snyder, S. H. (1990) *Science* **249**, 802-804.

23. Challis *et al.* (1993) *NeuroProtocols* **3**, 135-144.