Phosphatidate as a Molecular Link Between Depolarization and Neurotransmitter Release in the Brain

Abstract. *Phosphatidate, a neuronal phospholipid, stimulated the uptake of calcium by nerve terminals isolated from the striatum of rat brain. This effect was not produced by other phospholipids or glycolipids. Phosphatidate, but not other phospholipids, evoked the release of [^{3}H]dopamine from striatal synaptosomes. The magnitude of both effects was similar to that observed after chemical depolarization of the nerve terminals. These results show that phosphatidate is the only membrane lipid component that acts as a functionally competent ionophore and support the suggestion that phosphatidate may serve as a link between depolarization and neurotransmitter release in the brain.*

The release of neurotransmitters by the action potential is a key event in synaptic communication; neuronal depolarization activates an influx of calcium into the nerve ending, and the increase in intracellular calcium triggers the release of a neurotransmitter (*1*). Although the molecular mechanism that couples depolarization to calcium influx is obscure, it is known that synaptic transmission and other stimulus-secretion mechanisms are coupled with specific changes in two membrane components: phosphatidate (PA) and phosphatidylinositol (PI) (*2*). Larabee *et al.* (*3*) showed that electrical stimulation of sympathetic ganglia specifically increases the incorporation of $^{32}P_i$ into PI, but not into PA. This effect is blocked by *d*-tubocurarine, indicating that this PI effect is postsynaptic. However, the electrical stimulation of synaptosomal beds (*4*) or the application of acetylcholine to synaptosome preparations (*5*) results in a significant increase in the incorporation of ^{32}P into PA. Generally, changes in PI under these conditions are variable. Other studies have shown that the initial event in the PA effect is the breakdown of PI, probably to the D-1,2-*sn*-diglyceride, which is then converted to PA (*6*).

In general, the PI-PA effect has been localized in synaptic vesicles (*7*), although in one study (*8*) it was localized in synaptic membranes. Pickard and Hawthorne (*7*) concluded that the most important physiological event is diglyceride formation, which promotes the fusion of vesicle and plasma membrane for exocytosis. More recent reports suggest that PA may function as the physiological mechanism for regulating the calcium gate (*9*). In synaptic membranes, PA could be the coupling agent between depolarization and calcium influx. We tested the functional aspect of this possibility by evaluating the effect of PA on the transport of ^{45}Ca by nerve terminals (synaptosomes) and on the release of [^{3}H]dopamine.

Depolarization of synaptosomes by elevated concentrations of potassium triggers a net uptake of calcium, and this uptake is proportional to the degree of depolarization (*10*). We isolated synaptosomes from the striatal region of rat brain and compared the effects of potassium depolarization and exposure to PA on the transport of ^{45}Ca from the medium into the synaptosomes (*11*). The transport of calcium into the synaptosomes greatly increased when PA was added to the synaptosomes in a nondepolarizing medium (Table 1). The effect was similar to that produced by depolarization with 37 m*M* potassium. The maximal effect of adding PA was reached in 2 to 5 minutes, a time course similar to potassium- or glutamate-stimulated uptake (*12*). A PA concentration of $10^{-5}M$ also stimulated calcium uptake, but this effect was less than that produced by a $10^{-4}M$ concentration. Other lipids, including PI, phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, gangliosides, and sulfatides, did not increase synaptosomal calcium uptake when tested at a concentration of $10^{-4}M$. Thus, only PA functioned as a calcium ionophore at the synaptosomal membrane.

In order to determine whether calcium transported by PA is functionally equivalent to that taken up as a result of depolarization, we measured the release of [^{3}H]dopamine from perfused synaptosomes (*13*). Phosphatidate was incorporated into synaptosomes after loading with [^{3}H]dopamine in a calcium-free medium. The synaptosomes were washed and then perfused with a normal (3 m*M*) or a low (> 0.1 m*M*) calcium buffer. The effect of calcium-stimulated [^{3}H]dopamine release from PA-treated synaptosomes (Table 2) was similar to that induced by 44 m*M* K^+. Both the potassium- and PA-induced release were abolished when calcium was omitted from the perfusion medium, indicating that the releasing effect of these agents resulted from calcium influx. Of the naturally occurring membrane lipids, only PA stimulated [^{3}H]dopamine release. It is important to note that in the calcium-transport and neurotransmitter assays, PA was incorporated into the membranes in the absence of calcium. Thus, the ionophoric action was due to PA in the membrane, not to calcium PA in the aqueous phase.

These results demonstrate that PA is the only naturally occurring membrane lipid component that acts at the synapse as a functionally competent ionophore. The depolarization-induced increase in PA that results from the increased breakdown of PI was reported to be localized primarily in the synaptic vesicle fraction (*7*). However, a depolarization-linked increase in synaptic plasma membrane PA might not have been detected because (i) the bulk of the PI probably is located on the cytoplasmic surface of the synaptic membrane (as it is in other membranes) (*14*), and only a small pool of externally located PI would be involved in depolarization- and ionophore-linked mechanisms, and (ii) synaptic plasma membranes have an active PA phosphohydrolase (*15*) to metabolize and regulate the

Table 1. Stimulation of calcium uptake by phosphatidate (PA). Accumulation of ^{45}Ca was measured in synaptosomes isolated from rat brain striata (*11*). Calcium was added only after PA was incorporated into the membranes. Values represent mean ± standard error of the mean of five or six determinations, each performed twice.

Additions	Calcium accumulation (nanomoles per milligram of protein)
None	4.3 ± 0.3
PA ($10^{-4}M$)	10.3 ± 0.5*
None	4.9 ± 0.5
KCl (37 m*M*)	11.4 ± 0.7*

*Significantly different from unstimulated control ($P < .01$).

Table 2. Stimulation of neurotransmitter release by phosphatidate (PA). The release of [^{3}H]dopamine was studied in slices of rat brain striata (*13*). Calcium was added only after PA was incorporated into the membranes. Values are mean ± standard error of the mean of five or six determinations, each performed twice.

Additions	Dopamine release (percent of released fraction)
Ca alone	6.6 ± 0.5
PA ($10^{-4}M$) and Ca	11.1 ± 1.1*
K^+ (44 m*M*) and Ca	11.7 ± 1.0*
None	7.4 ± 0.5
PA, no Ca	7.2 ± 0.7
K^+ (44 m*M*), no Ca	8.1 ± 0.7

*Significantly different from unstimulated control ($P < .01$).

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levels of membrane PA. Thus, a depolarization-induced increase in PA should be small and transient.

In the bilayer model of the cell membrane, the phospholipids are oriented with their polar head groups near the membrane-water interface, and movement of phospholipids (flip-flop) is limited (*16*). This model does not allow for the rapid movement of complexes of calcium and PA through the membrane. However, calcium has been shown to convert acidic lipids from the bilayer configuration to a hexagonal phase (*17*). This conversion produces lipidic intramembranous particles that are not restricted to half of the bilayer (*17*), and these might function as ionophores.

An alternative possibility for the role of the PI effect in stimulus-secretion coupling is that the increased breakdown of PI results in the release of arachidonoyl residues (*18*). In the pancreas, this arachidonate is then converted to prostaglandins, which stimulate secretion (*18*). It is unlikely that arachidonate release is the basis of the PA effects observed in our study, because our results were obtained with dipalmitoylphosphatidate, which cannot be metabolized to arachidonate. This PA may be hydrolyzed to release palmitate, but we have found that palmitate does not alter synaptosomal calcium transport (*19*).

Our findings suggest an important role for PA in the mechanisms of psychoactive drug action. A variety of cationic drugs, including *d*-amphetamine and morphine, which increase neurotransmitter release, also inhibit PA phosphohydrolase (*2*). Thus, one of the mechanisms by which these drugs increase neurotransmitter release could involve a PA-related mechanism.

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11. Synaptosomal calcium uptake was measured, with a slight modification, by the method described in (*12*). Synaptosomes from rat brain striatum were suspended in a calcium-free buffer solution and incubated at 30°C for 15 minutes. Phosphatidate (dipalmitoyl or egg yolk suspended in water; Sigma Chemical Co., St. Louis, Mo.), or water as the control, was then added to give a final concentration of $10^{-4}M$. Two minutes later, a solution containing ^{45}Ca was added, and 5 minutes later uptake was halted. The synaptosomes were removed by filtration, and the amount of ^{45}Ca was determined and the potassium-stimulated calcium uptake was assayed as described in (*12*).
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13. Release of [^{3}H]dopamine from synaptosomes was determined with a perfusion apparatus similar to that described by L. P. Davies, G. A. R. Johnston, and A. L. Stephanson [*J. Neurochem.* **25**, 387 (1975)]. Striatal synaptosomes were incubated for 5 minutes at 30°C in a calcium-free Krebs-Ringer buffer with $10^{-7}M$ [^{3}H]dopamine. Phosphatidate or other phospholipids were added, and the incubation was continued for 5 minutes. The mixture was chilled, centrifuged, and the pellet obtained was washed twice with 0.32*M* sucrose and 5 m*M* Hepes, *p*H ≈ 7.8. The pellet was resuspended in the calcium-free buffer and approximately 2 mg of synaptic protein was collected on a Gelman glass fiber filter (minimum pore size 0.2 μm). The perfusion was at 4°C initiated with either calcium-free (< 0.1 m*M*) or calcium (3 m*M*) buffer at a rate of 0.5 ml per minute. After the first fraction (4 ml) was collected, the temperature was raised to 30°C. The effects of PA plus Ca^{2+} were significantly different from PA alone in three fractions. The data in Table 2 give the mean of the percent of the total released in these fractions. In some experiments, control synaptosomes were perfused with high K^+ (44 m*M*) buffer in the presence or absence of calcium. At the end of the experiment, the tissue was solubilized with 0.2 percent sodium dodecyl sulfate. The residual [^{3}H]dopamine comprised > 85 percent of the material released as judged by alumina chromatography.
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Two-Hundred-Million-Year-Old Chromosomes: Deceleration of the Rate of Karyotypic Evolution in Turtles

Abstract. *Cladistic analyses of chromosomal banding patterns from 48 species of cryptodiran turtles, combined with a fossil-based method for estimating rates of karyotypic change, show that karyotypic evolution was twice as fast and involved different types of rearrangements in Mesozoic turtles when compared to more recent forms. The deceleration in rate of karyotypic change is correlated with decelerated morphological change and is indicative of adaptive evolution. Comparisons of banded karyotypes reveal that some chromosomes have remained unchanged for at least 200 million years.*

Rates of karyotypic evolution vary tremendously both within and among major taxa of animals and plants (*1–9*). Accurate determinations of such rates are desirable for testing alternative models of chromosomal evolution because most models attempt to explain why the rates of chromosomal rearrangement incorporation differ among taxa. For example, one model suggests that taxa characterized by slow rates of karyotypic evolution, morphological evolution, and speciation possess large effective population sizes (N_e). Conversely, taxa with small N_e due to low vagility, territoriality, or social structuring (factors that promote inbreeding and population subdivision) are expected to experience rapid karyotypic, morphological, and speciation rates (*1–5, 10*).

An alternative hypothesis (*11*) suggests that natural selection directly favors chromosomal rearrangements when their phenotypic effects confer an adaptive advantage. The early stages of major adaptive radiations are thought to be characterized by rapid rates of karyotypic evolution; later, the rates of karyotypic evolution decelerate after the incorporation of adaptive gene sequences. The types of rearrangements incorporated also are thought to change through time. Early stages of the canalization process incorporate rearrangements (such as inversions and translocations) that alter gene arrangement (and presumably regulation), while later stages are more likely to involve rearrangements less likely to have phenotypic effects (heterochromatin additions, centric fusions). The two models (termed the deme size and canalization models) are testable because they offer different predictions as to rates of karyotypic evolution. The deme size

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