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# The muscarinic stimulation of phospholipid labeling in hippocampus is independent of its cholinergic input

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The enhanced incorporation of $^{32}P_i$ into two minor acidic phospholipids, phosphatidate and phosphatidylinositol, is a response characteristic of a number of effectors, including neurotransmitters and hormones, with their receptors on the cell membrane. In the nervous system, this is perhaps best documented in the case of acetylcholine action on brain synaptosomes, slices or on sympathetic ganglia (for review, see ref. 14). This 'phospholipid labeling effect' (PLE) appears to require tissue structural integrity since it is not observed in cell-free preparations. The PLE elicited by muscarinic agonists in nerve ending fractions[19] might be considered an exception. However, isolated synaptosomes retain considerable internal structure and metabolic viability, and as such may be considered as resealed anucleate neurons. Neurotransmitter receptors have traditionally been considered to be localized on the postsynaptic membrane, and a number of indirect approaches suggest that the neurotransmitter-directed PLE in pineal gland[21] and superior cervical ganglion[11] is associated with this site. In the case of synaptosomes, this conclusion is not readily made. For example, the assumption that added $^{32}P_i$ must first be converted to $\gamma$-$^{32}$P-ATP before phospholipids can be labeled suggests that intrasynaptosomal oxidative phosphorylation is an obligate step in the synaptosomal PLE (see ref. 19), an argument that would appear to favor a presynaptic localization. Furthermore, postsynaptic membranes are not prominent in the 'light' synaptosomal fraction shown to mediate the PLE in guinea pig brain fractions[20]. The case for a presynaptic site is however by no means conclusive. In a recent study, we concluded that calcium mobilization accompanies the muscarinic PLE (see ref. 5) in synaptosomes. The increase in calcium availability that parallels the PLE is at variance with the probable physiological role of presynaptic muscarinic receptors in cerebral cortex in reducing neurotransmitter release[15,22], by appearing to limit calcium availability[13]. That is, we would have anticipated that ac-

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tivation of a presynaptic receptor would be mimicked by reducing rather than by increasing calcium flux. The observed calcium effect prompted us to re-examine the inferred localization of the cholinergic receptors responsible for the synaptosomal PLE. In this report, we have experimentally broached this question by examining the role of cholinergic innervation on the acetylcholine (ACh)-induced PLE in synaptosomes derived from the hippocampus. The hippocampus supports a robust PLE and its sole cholinergic input from the septal nuclei can be readily disrupted by the placement of lesions in the fornix. As discussed below, the lesion is expected to cause degeneration of cholinergic presynaptic fibers, but should have little effect on the integrity of postsynaptic structures, and thus provide a means of further localizing the synaptosomal PLE.

Male guinea pigs (250-350 g) were anesthetized with pentobarbital (35 mg/kg) and placed in a stereotaxic frame (Kopf Instruments). Bilateral anodal electrolytic lesions of the fornix (0.5 mm posterior to bregma, 5.2 mm below brain surface and 1 mm lateral to the midline) were effected by passing a current of 8 mA for 30 sec. Eight to ten days after surgery, the guinea pigs were killed by stunning and exsanguination, the overlying cortical structures displaced by means of a blunt spatula, and the hippocampal formation (including subiculum, dentate gyrus and hippocampus proper) from both sides removed. The hippocampal formation (150-240 mg wet weight) was homogenized in 6 ml of 0.32 M sucrose and a nerve ending fraction ($P_2B$) prepared by conventional methods[8]. Lipid labeling from $^{32}P_i$ in the absence and presence of ACh ($10^{-4}$ plus $10^{-4}$ M eserine) was carried out by incubating aliquots of synaptosomes (200-400 $\mu$g protein) in a medium containing (final concentrations): 100 mM sodium glycylglycinate buffer (pH 7.4); 1 mM sodium pyruvate; 1 mM sodium fumarate; 1 mM $MgSO_4$; 0.5 mM $CaCl_2$; 0.1 mM $NaH_2PO_4$; and 60-150 $\mu$Ci of $^{32}P_i$ (carrier free) in a total vol. of 0.25 ml. Incubations were terminated after 45 min and lipids extracted and quantified essentially as described previously[20]. Choline acetyltransferase (ChAT) was determined by a radiochemical assay[6]. Acetylcholinesterase (AChE) activity was measured spectrophotometrically at 37 °C by the method of Ellman et al.[3], with the inclusion of $10^{-5}$ M tetraisopropylpyrophosphoramide to inhibit pseudocholinesterase activity[12]. The binding of [$^3$H]-quinuclidinylbenzilate (QNB) was measured by incubating 40-160 $\mu$g of protein for 60 min at 25 °C in the presence of 1 nM QNB (29.4 Ci/mmol)[25]. Reactions were terminated by rapid vacuum filtration of assay components through glass fiber filters (Whatman GF/B). Specific binding is defined as total binding minus binding in the presence of $10^{-6}$ M atropine. Glutamate decarboxylase (GAD) activity in synaptosomes (70–250 $\mu$g protein) was determined by measuring $^{14}CO_2$ evolution from sodium L-[1-$^{14}$C]glutamate. Each incubation contained (final concentrations): 2 mM sodium L-glutamate (0.28 mCi/mmol); 50 mM sodium phosphate buffer (pH 6.8); 1 mM dithiothreitol; 0.2 mM pyridoxal-5′-phosphate; 10 mg/ml BSA; 1 mM sodium arsenite and 0.5% (v/v) Triton X-100 in a total vol. of 0.1 ml. Reactions were terminated after 120 min and the $^{14}CO_2$ evolved at 37 °C measured as described previously[4]. Protein was determined by the method of Geiger and Bessman[7]. Results are expressed as mean $\pm$ S.E.M. Student's t-tests were used to evaluate differences in means of sets of data.

In unlesioned control animals, the inclusion of $10^{-4}$ M ACh stimulated the incorporation of $^{32}P_i$ into both phosphatidate (PhA) and phosphatidylinositol (PhI) by 86 and 55% respectively (Table I). Atropine ($10^{-5}$ M) completely blocked this stimulation of lipid labeling, while D-tubocurarine ($10^{-4}$ M) had no effect. This result is in accordance with our previous observations, and those of others, showing that the cholinergic PLE in cerebral cortex[19], caudate nucleus[1] and sympathetic ganglia[18] is muscarinic.

The extent and nature of changes in both basal and stimulated lipid labeling were not significantly affected by interruption of the cholinergic input. Thus, after placement of fornix lesions, synaptosomal fractions from the hippocampus showed mean stimulations of PhA and PhI labeling of 76 and 41 % respectively, on addition of ACh (Table I). In marked contrast, the fornix lesions resulted in a large (72%) reduction of ChAT, a presynaptic cholinergic marker enzyme[6], and a 56% reduction of AChE, an enzyme also considered to be presynaptic in the hippocampus[17,24]. Since several animals showed an almost total loss of ChAT activity, the partial loss following lesions is more likely to reflect an incomplete transection of fiber tracts, rather than the presence of a separate population of cholinergic neurons. The prevalence of muscarinic receptors present in the synaptosomal fraction, as determined by [$^3$H]QNB binding, was unchanged following the lesion, which suggests that these synaptosomal muscarinic receptors are not present on cholinergic nerve endings. Although it is possible that a small population of presynaptic muscarinic receptors may exist within the hippocampus[16,27] it seems unlikely that they contribute significantly to the PLE. GAD activity also remained unchanged after the fornix lesions, indicating that intrinsic GABAergic neurons had not been affected.

TABLE I

*The effect of fornix lesions on the phospholipid labeling effect, marker enzymes and muscarinic receptor binding in synaptosomal fractions from hippocampus*

ΔPhA and ΔPhI refer to per cent stimulation of labeling from $^{32}P_i$, by ACh. Units of measurement were as follows: ChAT, AChE and GAD: nmol/mg protein/min; QNB: pmol bound/mg protein; protein: mg/g wet weight. The numbers in parentheses refer to the number of separate experiments. Values for marker enzyme activities and QNB binding were also obtained for whole homogenates from both control and lesioned groups. These results indicated that alterations in the various parameters after fornix lesions closely paralleled those in the nerve ending fraction.

| | *Control* | | *Lesioned* | |
|---|---|---|---|---|
| ΔPhA | 86 ± 5 | (12) | 78 ± 4 | (15) |
| ΔPhI | 55 ± 6 | (12) | 42 ± 6 | (15) |
| ChAT | 1.52 ± 0.07 | (12) | 0.42 ± 0.06 | (17)* |
| AChE | 71.2 ± 6.3 | (9) | 31.3 ± 4.8 | (9)* |
| QNB | 0.98 ± 0.08 | (11) | 0.94 ± 0.06 | (10) |
| GAD | 2.31 ± 0.15 | (8) | 2.22 ± 0.14 | (13) |
| Protein | 10.3 ± 0.6 | (15) | 9.8 ± 0.2 | (17) |

* Statistically different from control group, $P < 0.001$. All other comparisons were not significant at the $P = 0.05$ level.

The dissociation of the synaptosomal PLE from the septal cholinergic input demonstrates that the muscarinic receptors coupled to stimulated PhA and PhI turnover are present on structures that are non-cholinergic in origin. Possible sites of localization of these cholinergic receptors include the dopaminergic, noradrenergic or serotonergic nerve endings shown to be cholinoceptive in other systems[2,10,13], or on dendrite-derived particles ('dendrosomes')[9].

While we cannot at present unequivocally identify the membrane locus of the PLE, the most parsimonious interpretation of our results is that in hippocampal synaptosomes, and by inference, in brain synaptosomes in general, the PLE occurs in a structure that is postsynaptic to the physiological site of the ACh release. In this respect, the postsynaptic location of the synaptosomal PLE is in accord with the inferred site of stimulated lipid labeling observed in sympathetic ganglia[11] and pineal gland[21].

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