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Sound Stimulates Labeling of Polyphosphoinositides in the Auditory Organ of the Noctuid Moth

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Abstract: The Noctuid moth possesses a simple auditory structure suitable for the investigation of biochemical correlates of sound stimulation *in vivo*. Stimulation with pulsed tones increased ^{32}P incorporation into polyphosphoinositides but not into ATP or other lipids. The effect was seen in the scoloparium (sensory structure) but not in the nodular sclerite, an adjacent nonsensory tissue. It was also not seen when the stimulus was a continuous tone, leading to adaptation of the action potential. **Key words:** Phospholipids—Phosphatidylinositol phosphate; biphosphate—Acoustic stimulation—Biopotentials.

Molecular changes concomitant with the electrophysiological events in stimulated auditory receptors are as yet unknown, at least partly for the lack of a suitable experimental model. For our purpose, an auditory structure is needed in which the electrophysiological detail of its response is sufficiently well defined and simple to permit a correlation with biochemical findings.

The ear of the Noctuid moth appears to meet these criteria. It is easily accessible and has a markedly simple morphology and electrophysiology. The sensory tissue, the scoloparium, contains two sensory cells along with a few supporting cells (Ghiradella, 1971). The sensory cells are true auditory receptors, of essentially similar physiological characteristics (Suga, 1961; Adams, 1971; Roeder, 1971). Only two types of bioelectric activity are induced by sound stimulation in these primary sensory cells: the generator or receptor potential, tantamount to the transduction event in the dendritic ends, and the action potential in the axonal segments. The preparation thus seemed well suited for an investigation of the role of polyphosphoinositides in hearing processes.

The polyphosphoinositides, phosphatidylinositol phosphate and phosphatidylinositol bisphosphate[1], are quantitatively minor phospholipids of eukaryotic cells, but the turnover of their monoesterified phosphate groups is very rapid, as shown by ^{32}P radiotracer techniques. The rate of this turnover in neural tissue, the tissue in which these lipids primarily occur in mammals, is not invariant. Excitation of a variety of nerves and axons leads to changes in polyphosphoinositide metabolism (Birnberger et al., 1971; Schacht and Agranoff, 1972; Tret'jak et al., 1977; Abdel-Latif et al., 1978), and a phosphorylation and dephosphorylation cycle has been suggested as being associated with membrane permeability changes in axonal conduction (Griffin and Hawthorne, 1978; Kai and Hawthorne, 1969). In insects, polyphosphoinositides seem to have a widespread tissue distribution which includes nerves and sensory systems (Bridges, 1973; Kilian and Schacht, 1979).

We have previously proposed a mechanism for the transduction of acoustic energy into a generator potential by regulation of phosphorylation-dephosphorylation reactions in the receptor membrane by sound energy (Kilian and Schacht, 1977). The polyphosphoinositides were suggested as candidates for this scheme, also, because studies with aminoglycosidic antibiotics suggested a specific role for these lipids in auditory phenomena. A change in turnover of these lipids after administration of

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[1] 1-(3-sn-Phosphatidyl)-D-*myo*-inositol 4-phosphate and 1-(3-sn-phosphatidyl)-D-*myo*-inositol 4,5-bis(phosphate).

neomycin or related ototoxic compounds has been shown in the cochlea of the guinea pig to correlate with a decrease in the microphonic potential (Stockhorst and Schacht, 1977; Nuttall et al., 1977).

We present here evidence that the ear of the Noctuid moth is a suitable experimental model and, further, that sound stimulation leads to specific changes of polyphosphoinositide labeling.

MATERIALS AND METHODS

Experiments were performed on laboratory-reared, male *Agrotis ypsilon* at 25°C in a soundproof room. The methodology has been described elsewhere (Kilian and Schacht, 1979). Briefly, the moths were temporarily anesthetized in ether vapor and their wings and legs were removed. The body of the moth was then pinned to a platform under a stereomicroscope so that the ear on the right side was clearly exposed. After denuding the area around the ear, 7 μl of carrier-free [^{32}P]orthophosphate was injected into the air space below the epimeron adjacent to the tympanic air sac. Only tissues on the right side were analyzed. Other routes of injection of isotope lead to less efficient labeling of phospholipids in the ear. At various times following injection of the isotope, moths were killed by an injection of 10 μl of glutaraldehyde followed immediately by 30 s of microwave irradiation at 1200 W (Litton 70/50). This procedure was most reliable for arrest of enzymatic activity and prevention of postmortem breakdown of the polyphosphoinositides. The ear tissues were dissected by hand and the scoloparium was removed, with a small piece of tympanic membrane attached to facilitate handling. Ten to twelve tissues were pooled for each analysis and a homogenate of guinea pig brain was added to the ear tissues as a source of carrier lipids. Phospholipids were extracted into acidified chloroform-methanol, separated by thin-layer chromatography, and located by autoradiography (Schacht, 1978). [^{32}P]Nucleotide phosphate was analyzed by absorption on charcoal (Crane and Lipman, 1953).

For sound stimulation, a 75 dB (re: 20 μN/m^2), 40 kHz tone was delivered by a piezoelectric speaker (Eardrum, Los Angeles, California). One group of moths was stimulated from the time of injection of the isotope until they were killed ("tone") while another group were kept in silence ("no tone"). Prior to the selection of the acoustical stimulus, a threshold curve for the tympanic organ of *Agrotis ypsilon* had been obtained. The insect is most sensitive to ultrasonic frequencies, with highest sensitivity at 40–60 kHz (Kilian and Schacht, 1977).

The statistical procedures used are described by Schefler (1969).

RESULTS

Lipid Labeling

Two tissues of the moth ear were analyzed: the scoloparium, containing the sensory cells, and the nodular sclerite, a strip of cuticle adjacent to the tympanic membrane. Phosphatidylinositol phosphate and bisphosphate were most rapidly labeled, reaching maximal ^{32}P incorporation at about 30 min. Other quantitatively major phospholipids (phosphatidylserine, phosphatidylinositol, and phosphatidic acid) showed a slower rate of ^{32}P incorporation (Fig. 1).

Sound Stimulation

The effect of sound stimulation on ^{32}P labeling in the scoloparium was determined in five experiments (Table 1). The variability of the data was small when experiments were conducted in duplicate at the same time (Exp. 4 a and b; 5 a and b) while the variability between experiments was larger. This variability may be due to seasonal variations since these experiments were conducted over a year.

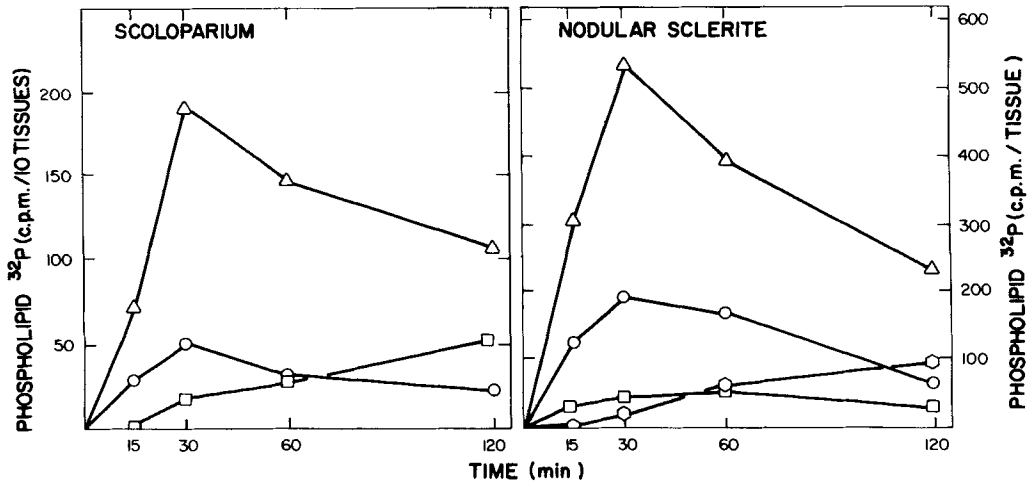

FIG. 1. Time-course of ^{32}P-labeling. One hundred μCi of $^{32}P_i$ (carrier-free, in a volume of 7 μl) was injected into the moth as described in Methods. Moths were killed at times indicated, and 10 tissues were pooled for each point. (□), phosphatidic acid; (left, □; right, ⬡) phosphatidylinositol, phosphatidylserine; (○), phosphatidylinositol phosphate; (△), phosphatidylinositol phosphate, bisphosphate.

TABLE 1. *^{32}P-labeling of the Noctuid moth ear (Scoloparium)*

	ATP[a]		PhIP[b]		$PhIP_2$[c]	
Exp.	No tone	Tone	No tone	Tone	No tone	Tone
1	18	17	6.0	12	19	36
2	19	17	2.1	3.6	5.5	10
3	10	13	5.3	8.7	15	27
4a	12	11	7.2	6.4	19	20
b	13	13	9.3	7.3	19	16
5a	15	18	2.7	5.1	5.5	8.0
b	16	18	4.3	4.4	7.3	8.5

Moths received 150 μCi of ^{32}P (carrier-free, vol. = 7 μl) and were killed after 30 min (15 min in exp. 5). "Tone" groups were stimulated during this time with a pulsed tone signal (40 kHz, 75 dB [re: 20 $\mu N/m^2$], 50 ms on, 1 cycle/s). Each value is from a pool of 10 to 12 tissues. Significance of differences between "no tone" and "tone" groups by χ^2 ranking: $p < 0.035$ for polyphosphoinositides.

[a] [^{32}P]Nucleotide phosphate as percent of total soluble ^{32}P.

[b] [^{32}P]Phosphatidylinositol phosphate as percent of [^{32}P]nucleotide phosphate.

[c] [^{32}P]Phosphatidylinositol bisphosphate as percent of [^{32}P]nucleotide phosphate.

Seasonal differences in auditory studies were reported early by Yerkes (1905) and recently by Sewell et al. (1978). Sound stimulation did not significantly affect the labeling of ATP from $^{32}P_i$, but most experiments indicated an increase in ^{32}P incorporation into phosphatidylinositol phosphate and bisphosphate. Analysis of the data by χ^2 ranking showed a significant difference between "tone" and "no tone" groups for the polyphosphoinositides.

In all of the experiments, labeling in the nodular sclerite was also measured. Since no active role in the hearing process has been ascribed to this structure, comparison of labeling in the two tissues in response to sound was made (Table 2). For each compound in each tissue, labeling in the presence of sound was expressed as the percentage of labeling without sound; i.e., a value of 100% indicates no effect of sound. Clearly, acoustic stimulation leads solely to changes in polyphosphoinositide labeling in the scoloparium. Labeling in the nodular sclerite remained unchanged with stimulation.

DISCUSSION

The increased labeling of polyphosphoinositides in the scoloparium during acoustic stimulation should be due to an enhanced rate of the turnover of the monoester phosphate groups of these lipids. While this conclusion cannot be reached unequivocally from our experimental data, it is supported by the fact that labeling of the precursor, ATP, was not stimulated. It is further supported by the finding that other labeled phospholipids in the scoloparium (phosphatidylinositol plus phosphatidic acid and phosphatidylserine) were not affected by sound. In addition, increased *de novo* synthesis can probably be ruled out since the contribution of this pathway to polyphosphoinositide labeling in the moth is very small for brief incubation times (Kilian and Schacht, 1979).

The increased labeling of polyphosphoinositides in only the sensory tissue suggests a specific correlation of these changes with a hearing-related process. Additional clues about the physiological role

TABLE 2. *Effects of tone presentation on ^{32}P labeling in scoloparium and nodular sclerite*[a]

	Scoloparium	Nodular sclerite
ATP	105 ± 15	102 ± 23
Phosphatidylinositol phosphate	142 ± 50[c]	94 ± 24
Phosphatidylinositol bisphosphate	143 ± 40[d]	91 ± 24
Other lipids[b]	107 ± 43	98 ± 29

[a] Labeling in the presence of stimulus as percent of labeling in its absence. Numbers are means ± S.D. of five experiments.

[b] Labeled phosphatidic acid, phosphatidylinositol, and phosphatidylserine.

[c] The effect in scoloparium differs from nodular sclerite by $0.02 < p < 0.05$ (Wilcoxon) and $p = 0.06$ (2-sample t-test). [d] The effect in scoloparium differs from nodular sclerite by $p < 0.01$ (Wilcoxon) and $p = 0.02$ (2-sample t-test). Other differences not significant.

are provided by comparing the effect of different tone stimuli. In the above experiments, the stimulus was a pulsed tone which initiates both generator and action potential activity in the auditory receptor of the moth. A continuous tone leads to rapid adaptation of spike activity, leaving the generator potential as the only bioelectric event (Adams and Belcher, 1974). Two preliminary radiotracer experiments indicate that the increased labeling observed with a pulsed tone is not seen with a continuous tone as a stimulus. In the latter case, average phosphatidylinositol phosphate was 2.9% (no tone) and 2.7% (continuous tone) of labeled ATP, while phosphatidylinositol bisphosphate was 11% and 10%, respectively. Therefore, the changes in polyphosphoinositide labeling with stimulation by pulsed tone appear to correlate with spike activity in the acoustic receptor of the Noctuid moth rather than with generator potential activity.

These data are consistent with the hypothesis that polyphosphoinositides play a role in events related to neural excitation. Previous studies testing this hypothesis were carried out under nonphysiological conditions; e.g., isolated nerves and axons were presented with an inadequate stimulus (electrical stimulation). Such studies yielded conflicting results about the turnover of polyphosphoinositides (Salway and Hughes, 1972; White and Larrabee, 1973; White et al., 1974). Our study is a demonstration of changes in polyphosphoinositide labeling upon presentation of an adequate stimulus in an *in vivo* experiment. In addition, we have demonstrated that the auditory organ of the Noctuid moth can be used to analyze molecular events in hearing. The simplicity of this ear allows us to correlate electrophysiological, biochemical, and pharmacological investigations and makes it a suitable model for the study of auditory processes.

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