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(17). The resulting instrument, however, appears to have broad application in the measurement of small motions since it uses a noncontact, optical method having high sensitivity, good spatial resolution, broad bandwidth, wide dynamic range, and high tolerance of background displacements of the target surface (18). Furthermore, in contrast to many vibration detectors, it is relatively simple to construct, portable, easy to operate, and inexpensive. In order to adapt the instrument to other uses, only slight changes (18) are required to maximize sensitivity, bandwidth, spatial resolution, or background movement rejection at the expense of one or more of the others.

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- For example, by using this technique to measure the displacement of a thin, aluminized Mylar diaphragm stretched over an enclosed air volume, a d-c coupled microphone or a sensitive barometer could be made. Such an instrument would not be limited at low frequencies by diaphragm capacitance as would be a conventional condenser microphone.
- Photodiode output current is proportional to total power (watts) incident on the photodiode. Since the illuminated area of the photodiode is constant, output current is proportional to irradiance, or power per unit area.
- The integral is the formula for the area of a circle between two lines drawn perpendicular to the circle's diameter.
- When the detector was set up in its most sensitive position with the shadow boundary at $y_0 = 0$, accurate measurement of THD was impossible because of the difficulty of producing a target motion signal with less distortion than the detecting instrument. However, as the shadow boundary was shifted away from $y_0 = 0$, detector distortion increased and measurement of THD was possible. The THD measured by a spectrum analyzer (Nicolet Scientific, Miniubiquitous 444A) was less than predicted. There are two reasons for this, both a result of simplification of the theory of operation to permit easier understanding and analysis. First, the shadow boundary is not sufficiently sharp because the light source is not a point. Second, some light from outside the area seen through the aperture of the light guide reaches the photodiode via internal reflections in the light guide tube. These two factors moderate the distortion-producing edge effects by making changes in illumination of the photodiode more gradual than predicted.
- The minimum detectable motion of the target surface depends on both the reflective properties of the surface and on the signal bandwidth. If the target is diffusely scattering rather than specular, much of the light striking the target is scattered off beyond the admittance angle of the light guide and, therefore, a given change in illuminated area produces less change in irradiance of the photodetector and hence less change in output. Signal bandwidth is also a major consideration since background noise is proportional to bandwidth. Over the full motion-bandpass of the instrument (10 to 120,000 Hz) the equivalent input noise motion (that is, the target motion that would produce the measured output noise if the detector and preamplifier were noiseless) is $0.043 \mu\text{m}_{\text{rms}}$ (where rms is root mean square) for a specular target (front-surface mirror) and $0.7 \mu\text{m}_{\text{rms}}$ for a scattering target (white, nonglossy paper). For insect cuticle, the equivalent input noise motion will depend on the properties of the cuticle but will normally lie between these values. Reflectivity of an insect's surface can be increased by attaching a small piece of aluminized Mylar tape to the insect. Under optimal conditions (specular target) amplitudes of motion on the order of 0.1 nm have been measured, at a signal-to-noise ratio of ~ 10 dB, by means of lock-in detection with an effective analysis bandwidth of 0.01 Hz.
- P. B. Brown, *IEEE Trans. Biomed. Eng.* **21**, 428 (1974). The sensitivity of this detector, as determined by static displacement of the platform with a micrometer, was $21.6 \text{ mV}/\mu\text{m}$.
- Since the upper frequency limit of the stereo tape recorder (Uher 4400 Report Stereo IC) is approximately 15 kHz (± 1.5 dB from 50 to 15,000 Hz) and insect sounds frequently extend to ultrasonic frequencies, the signal is also recorded on an instrumentation tape recorder (Lockheed 417, not shown in Fig. 1b), which has a frequency response extending to 100 kHz (± 1 dB from 1 to 100 kHz when recorded at 76 cm/sec and replayed at 9.5 cm/sec for analysis). The airborne sound associated with the vibration signal being studied is also recorded on the instrumentation recorder by means of an ultrasonic microphone (± 1.5 dB from 1 to 100 kHz).
- Enaphalodes rufulus* (Cerambycidae).
- Each pulse in Fig. 2, trace d, consists of several cycles of damped oscillation at about 30 kHz. A 30-kHz sine wave having the maximum amplitude of any pulse in trace d would have a sound pressure at the microphone of approximately 75 dB sound pressure level, which corresponds to a peak-to-peak amplitude of molecular air motion of about 4 nm. Since the sound was recorded at a distance of 38 mm and the beetle can be approximated as a radially pulsating cylinder 7 mm in diameter, an impulsive motion of the cuticle of ~ 20 nm would be necessary to produce the airborne impulse.
- Amplitudes of motion on this order can be measured with this instrument by using signal processing techniques such as lock-in detection, signal averaging, or autocorrelation, but these techniques are infeasible when applied to insect disturbance sounds due to the broadband, transient and unpredictable nature of these sounds.
- Radiation efficiency is low when the dimensions of the vibrating source are small compared to the wavelength of the radiated sound [see P. M. Morse, *Vibration and Sound* (McGraw-Hill, New York, 1948)]. Thus, although the maximum energy of vibration is at about 1 kHz in Fig. 2, trace a, little sound is radiated at this frequency because the beetle is only ~ 7 mm wide compared to a 1-kHz wavelength in air of ~ 340 mm. On the other hand, the frequency of maximum airborne energy is at about 30 kHz. At this frequency the wavelength in air is ~ 11 mm and so coupling of vibration energy to air is more efficient.
- Many different insects produce sounds when handled or otherwise disturbed. It has been proposed that these disturbance sounds serve to startle or warn potential predators [P. T. Haskell, *Insect Sounds* (Quadrangle Books, Chicago, 1961); T. Eisner, D. Aneshansley, M. Eisner, R. Rutowski, B. Chong, J. Meinwald, *Psyche* **81**, 189 (1974); T. Bauer, *Z. Tierpsychol.* **42**, 57 (1976)].
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- Specifications depend on the configuration chosen and on the associated electronics. A more complete description of the apparatus will be published elsewhere (W. M. Masters, in preparation). For the instrument described here, maximum sensitivity to motion is $134 \text{ mV}/\mu\text{m}$ with a broadband noise of $5.8 \text{ mV}_{\text{rms}}$, the target area seen is $\sim 0.14 \text{ mm}^2$, the 3-dB motion-band pass is 10 to 120,000 Hz, the dynamic range is > 100 dB, and background motion of the target can be up to $80 \mu\text{m}$ for ≤ -1 -dB change in output.
- Supported in part by an NSF predoctoral fellowship and a Bache Fund stipend (to W.M.M.), and NIH grant AI-02908 and NSF grant BMS 76-15084 (T. Eisner). The insects were identified by E. R. Hoebeke and the insect identification service of the U.S. Department of Agriculture. I thank the director and staff of the Archbold Biological Station, Lake Placid, Fla., where the recordings of insect sounds were made, R. R. Capranica for the loan of various pieces of electronic equipment, and D. Aneshansley, R. R. Capranica, T. Eisner, and A. J. M. Moffat for their comments on the manuscript.

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Synaptic Phosphoproteins: Specific Changes After Repetitive Stimulation of the Hippocampal Slice

Abstract. *Repetitive stimulation (100 pulses per second for 1 second) of the Schaffer collateral-commissural system of the rat hippocampus induces long-term potentiation of synaptic strength and produces significant changes in the subsequent endogenous phosphorylation of a 40,000-dalton protein from synaptic plasma membranes. This effect is not observed after stimulation in calcium-deficient media or after stimulation at the rate of one pulse per second for 100 seconds. These findings provide evidence that repetitive synaptic activation can alter the phosphorylation machinery of the synaptic region and suggest a biochemical process which may be involved in the production of neuronal plasticity.*

Historically, synaptic transmission has been analyzed in terms of biochemical and physiological events that take milliseconds to transpire. Recently it has become evident that, under some circumstances, the efficacy of the transmission process can be modified for periods ranging from minutes to months (1). There is evidence that phosphoproteins may be involved in such synaptic events. Protein phosphorylation can occur within seconds and is known to persist for at

least minutes (2); this alone makes the process attractive as a candidate for the biochemical substrate of relatively long-lasting changes in synaptic efficacy. Furthermore, a number of studies have provided evidence linking the phosphorylation machinery to synaptic events. Protein kinases, protein phosphatases, and their substrates have all been found in fractions enriched in synaptic plasma membranes (SPM's) (3). Other studies have shown that electrophysiological

and pharmacological manipulations of several putative neurotransmitter systems influence the concentrations of cyclic nucleotides in neural tissue (4); and Johnson *et al.* (5) and Ueda *et al.* (6), using quantitative gel autoradiography, demonstrated that the endogenous phosphorylation of at least two SPM proteins was enhanced in the presence of adenosine 3',5'-monophosphate (cyclic AMP). However, it has not been shown that changes in specific phosphoproteins accompany activation of a functional synaptic system. The present experiments were intended to provide evidence pertinent to this issue. Specifically, we have assayed the endogenous phosphorylation of proteins after repetitive stimulation of the hippocampal slice under conditions that produce semipermanent changes in synaptic efficacy.

Slices of rat hippocampus were prepared as previously described (7). About 1 hour after preparation, stimulation electrodes were placed in the stratum radiatum near the CA1-CA2 border in a position where they would activate the massive Schaffer and commissural afferents to the apical dendrites of the pyramidal cells (8). Pretetanus voltages (usually 5 to 20 V) were selected such that a single pulse would evoke a 1- to 2-mV "popu-

Table 1. Stimulation-dependent alterations in phosphorylation of specific SPM components. These data summarize the effects of stimulation at a rate of 100 pulses per second for 1 second in 20 experiments. The bands listed include only those which demonstrated detectable incorporation of label in ten or more of the 20 experiments. Quantification of the incorporation of label into specific bands was based on densitometric scans of autoradiographs. (These values were in good agreement with radioactivity profiles obtained from scintillation counts made of 1-mm slices of the gels.) Ratios between experimental and control values were determined for all detectable bands after normalization to control on the basis of total incorporation (as reflected by the densitometric values.) Percentage change is the average ratio for the 20 experiments. *N*, number of instances in which the stimulated value was greater (+) or less (-) than control.

Molecular weight	Percentage change (mean)	<i>N</i>	
		+	-
112,000	+3.6	10	4
85,000	-1.8	6	6
80,000	+7.9	12	5
68,000	+3.4	11	9
62,000	-0.9	5	8
53,000	+8.6	16	4*
50,000	-0.7	10	10
45,000	-3.2	4	7
40,000	-25.9	1	19†
33,000	+3.7	6	6
27,000	+16.0	15	2‡

**P* ≤ .012. †*P* ≤ .001. ‡*P* ≤ .002; two-tailed binomial test.

lation spike" in the cell layer. The pathway was then stimulated with biphasic constant-voltage pulses either for 1 second at 100 pulses per second or for 100 seconds at one pulse per second. Repetitive stimulation of this system at 100 pulses per second reliably produces a 200 to 1000 percent increase in the population spike and a 10 to 50 percent increase in the extracellular excitatory postsynaptic potentials recorded from the zone of Schaffer termination, and these changes persist indefinitely (9). This effect, long-term potentiation, is not obtained after stimulation at one pulse per second (10).

Molecular weight	Percentage change (mean)	<i>N</i>	
		+	-
53,000	-4.6	3	9
40,000	+4.0	8	4
27,000	-4.9	5	7

Two minutes after the cessation of high-frequency stimulation, the stimulated slice and an unstimulated paired control were transferred to separate solutions of 0.32M sucrose at 0° to 4°C. The tissue was maintained at this temperature throughout the isolation procedure. Similarly treated slices were pooled, and SPM fractions were prepared by a slight modification of previous methods (11). The SPM fractions were assayed for endogenous phosphorylation (12) and samples were then run on the exponential polyacrylamide gel system of Kelly and Luttges (13). The gels were stained for protein with Coomassie blue, dried, and exposed to Kodak RP Royal X-Omat film for 3 to 5 days. Autoradiographs were scanned on a Beckman 25 spectrophotometer.

Because changes produced in the phosphorylation machinery by treatments *in vivo* are reflected in a subsequent assay of endogenous phosphorylation (14, 15), we hypothesized that if repetitive stimulation of the hippocampus induces persistent changes in phosphorylation, then we might be able to detect these changes with a subsequent "post hoc" assay of endogenous phosphorylation. The results of a typical experiment (Fig. 1) indicate that repetitive, high-frequency stimulation does produce changes in the endogenous

phosphorylation of specific proteins. It was evident from visual inspection of the autoradiograph that a band of protein (16) with an apparent molecular weight of approximately 40,000 (40K) incorporated less label in the stimulated slices than in the paired controls. Detailed analysis of the protein bands from 20 separate experiments revealed a large, stimulation-dependent decrease in the 40K band in 19 of 20 experiments (Table 1). This effect could be reliably observed by visual inspection of the autoradiographs. Smaller and less consistent increases were detected in bands of protein with minimum molecular weights of 27K and 53K (Table 1).

We next determined whether synaptic activation was required for these effects. Accordingly, we repeated the experiment using slices that were incubated in media from which the calcium had been omitted. The presynaptic volley is largely unaffected by this condition, but the postsynaptic response is completely eliminated (10), presumably because of the absence of the depolarization-triggered calcium influx required for transmitter release. In seven separate experiments we observed no evidence of a decrease in the phosphorylation of the 40K protein; in fact, there appeared to be a tendency for stimulation to increase the

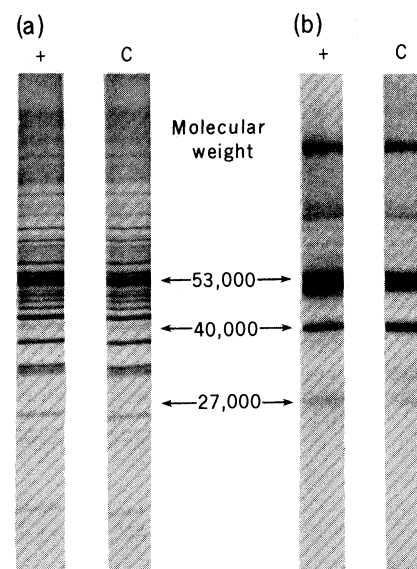


Fig. 1. Polyacrylamide gel depicting the effects of repetitive stimulation on phosphorylation of specific SPM components. Symbols: +, stimulated sample; C, control sample. (a) The protein staining pattern obtained with Coomassie blue in the 7.5 to 20 percent gel. (b) Autoradiograph showing the bands that incorporated label. The molecular weight estimations were based on comparisons with the mobilities of standards of known molecular weight. Molecular weight values shown indicate bands in which significant stimulation-dependent changes in phosphorylation were observed (see Table 1).

incorporation of radioactivity. No significant changes were observed in any of the other protein bands that we were able to analyze. Finally, we determined whether the change in the 40K protein was found only after repetitive high-frequency stimulation or instead was a general correlate of synaptic transmission. Accordingly, we performed 12 additional experiments in normal medium with 100 pulses delivered at a rate of 1 pulse per second, and subsequently assayed for endogenous phosphorylation; as shown in Table 2, such stimulation had no measurable effects on the 40K material.

Thus, high-frequency stimulation decreased the endogenous phosphorylation of a protein band (minimum molecular weight of 40,000), but low-frequency stimulation or stimulation in the absence of synaptic transmission had no such effect. The decrease after high-frequency stimulation suggests a number of possibilities about the mechanism of the stimulation-dependent change. Changes in cyclic AMP are probably not involved since neither this nucleotide nor the kinase it activates (17) have any significant effect on the endogenous phosphorylation of the 40K protein. The short time period of the assay (20 seconds) and the fact that the difference can be detected in a 5-second assay (data not shown) argue against the action of phosphatases because these enzymes generally display detectable activity only with significantly longer incubation periods (2). Thus it seems likely that stimulation produces either an increase in the phosphorylation of the protein, hence fewer sites would be available for labeled phosphate in the subsequent post hoc assay, or a decrease in the activity of a specific kinase, and therefore less labeled phosphate is transferred in the post hoc assay. Whatever the mechanism, these data indicate that repetitive stimulation of the type which induces long-lasting changes in synaptic efficiency also changes the endogenous phosphorylation of specific synaptic membrane phosphoproteins. Beyond providing a biochemical correlate of long-term potentiation, these findings provide evidence that the transmission process, under some circumstances, alters specific phosphoproteins of synaptic regions.

Although we have not identified the factors responsible for the stimulation-dependent change in the phosphorylation of the 40K protein, the dependence on calcium suggests that (i) the release of transmitter is the triggering event, or (ii) the influx of calcium associated with the transmission activates a kinase. With respect to the latter idea, calcium-sensitive

kinases have been identified in brain and other tissue (18), and we have found (17) that addition of one of these, phosphorylase b kinase (E.C. 2.7.1.38), to our assay results in a highly selective phosphorylation of the 40K band. This suggests that changes in intracellular calcium associated with repetitive stimulation may alter the 40K protein by way of activation of phosphorylase b kinase.

The appearance of the 40K effect after stimulation at 100 pulses per second, but not 1 pulse per second, suggests that this phosphorylation change may somehow be linked to the production of long-term potentiation. If so, it would represent an important insight into the biochemical mechanisms available to the brain to achieve lasting changes in synaptic strength, and as such may have implications for the understanding of the functional plasticity (for example, learning) which is so characteristic of the mammalian central nervous system.

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8. Neuroanatomical studies show that the Schaffer collateral and commissural afferents constitute the great majority of the fibers traveling through and terminating within the proximal dendritic zones of the pyramidal cells (that is, in the regions in which the stimulating and recording electrodes in the present study were located). The transmitters used by these synapses have not been conclusively identified but an increasing body of evidence implicates an acidic amino acid, most probably glutamic acid, for this role. The nerve terminals in this region possess a high affinity uptake system for acidic amino acids [L. Iversen and J. Storm-Mathisen, *Acta Physiol. Scand.* **96**, 22A (1976); J. Nadler, K. Vaca, W. White, G. Lynch, C. Cotman, *Nature (London)* **260**, 538 (1976)] and studies of hippocampal slices perfused locally show that isotopically labeled glutamic acid, but not aspartic acid, is released during synaptic activation in a calcium-dependent fashion that is essentially identical to that in the present study (A. Wieraszko and G. Lynch, *Brain Res.*, in press). The target dendritic regions of the Schaffer-commissural system are extremely sensitive to iontophoretically applied glutamic acids [H. Spencer, V. Gribkoff, C. Cotman, G. Lynch, *Brain Res.* **105**, 471 (1976)]. Catecholamine afferents to the pyramidal cell regions are very sparse and do not appear to follow the trajectory of the Schaffer-commissural axons [R. Moore, in *The Hippocampus*, K. Pribram and R. Isaacson, Eds. (Plenum, New York, 1975), vol. 1, p. 215]; it is therefore unlikely that noradrenergic fibers contribute to the effects reported here.
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12. Samples were incubated for 5 minutes at 30°C in 40 μ l of assay buffer at a protein concentration of 0.5 mg/ml. The reaction was initiated by the addition of 10 μ l of γ -³²P-labeled adenosine triphosphate (10 μ Ci; 20 μ M final concentration) in assay buffer. The reaction was terminated after 20 seconds by the addition of 30 μ l of a sodium dodecyl sulfate (SDS) solution, resulting in a final concentration of 2.3 percent SDS (weight to volume), 5 percent β -mercaptoethanol (by volume), 62.5 mM tris-HCl, 10 percent glycerol (by volume), pH 6.8. Kinetic studies indicate that, under these assay conditions, incorporation of label reflects the initial velocity of the phosphorylation reaction. Protein phosphorylation, as assayed here, refers only to changes in incorporation of label into electrophoretic bands, and no assumptions are made about the activity of kinases or phosphatases.
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