Cellular and Molecular Neurobiology, Vol. 5, No. 4, 1985

Activity-Related Changes in Protein Phosphorylation in an Identified *Aplysia* Neuron

Mark Schaefer,[1,2] Paul D. Shirk, [1,3] Daniel L. Roth,[1] and Philip H. Brownell[1]

Received January 14, 1985; revised May 15, 1985; accepted July 17, 1985

KEY WORDS: protein phosphorylation; peptidergic neurons; cyclic AMP; phosphodiesterase inhibitor; neuronal excitability.

SUMMARY

1. The relationship between long-term electrical activity and protein phosphorylation was investigated in single, identifiable neurons in the abdominal ganglion of *Aplysia californica* by the intracellular injection of radiolabeled ATP followed by sodium dodecyl sulfate (SDS) gel electrophoresis.

2. Natural and pharmacological treatments that alter the impulse activity of neurons L6 and R15 for prolonged periods did not appear to affect the phosphorylation of most of the 15 major phosphoproteins examined in these cells.

3. Long-term excitation of L6 induced by the phosphodiesterase inhibitor IBMX correlated with phosphorylation of a 29,000-dalton protein. Long-term inhibition of L6 induced by afterdischarge of peptidergic bag-cell neurons appeared to cause dephosphorylation of a 29,000-dalton protein.

4. Burst augmentation of R15 induced by bag-cell afterdischarge did not cause detectable changes in the phosphorylation of the major proteins we examined.

5. These data are consistent with other studies of neural and nonneural tissues which have found a correlation between activity and the level of phosphorylation of a 29,000-dalton protein.

[1] Department of Zoology, Oregon State University, Corvallis, Oregon 97331.
[2] To whom correspondence should be addressed at Department of Biological Sciences, Stanford University, Stanford, California 94305.
[3] Present address: Insect Attractants, Behavior, and Basic Biology Research Laboratory, USDA, A.R.S., Gainesville, Florida 32604.

0272-4340/85/1200-0321$04.50/0

INTRODUCTION

Prolonged bursts of impulse activity (hereafter referred to as afterdischarge) in the peptidergic bag-cell neurons of the abdominal ganglion of *Aplysia californica* results in the release of several peptide transmitters which strongly influence the excitability of other neurons in the ganglion. One of these transmitters, egg-laying hormone (ELH), appears to mediate a prolonged excitatory effect on neuron R15 (Branton, 1978), while another transmitter, α-bag-cell peptide (α-BCP), appears to inhibit neuron L6 (Rothman *et al.*, 1983). Similar long-lasting effects on the excitability of molluscan neurons can be induced by the bath application of cyclic nucleotide derivatives and phosphodiesterase inhibitors (Levitan and Norman, 1980; Kupfermann, 1980; Levitan and Adams, 1981; Siegelbaum and Tsien, 1983; Levitan *et al.*, 1984), suggesting that cyclic nucleotide-dependent protein phosphorylation may be involved in mediating changes in the electrical activity of these cells. Because bag-cell afterdischarge has essentially opposite effects on R15 (long-term excitation or burst augmentation) and L6 (long-term inhibition), we reasoned that a quantitive comparison of protein phosphorylation in these two neurons might reveal cell-specific phosphoproteins involved either in regulating excitability or in modulating the long-term metabolic responses to the electrical activity of these cells.

Since the original suggestion that protein phosphorylation might be involved in regulating neuronal electrical activity (Kuo and Greengard, 1969; Greengard, 1978), studies using whole brain tissues, nerve-cell cultures, and fractured-cell preparations have shown that phosphorylation of specific proteins correlates with pharmacological treatments that alter neural activity (reviewed by Nestler *et al.*, 1984; Nestler and Greengard, 1983; Kennedy, 1983). Recently, Lemos *et al.* (1982) took advantage of the favorable properties of the molluscan nervous system to examine phosphorylation in single, intact neurons. In this study we used the same approach in an attempt to identify changes in protein phosphorylation in two identifiable cells whose activity is modulated in an opposite fashion by peptides released during bag-cell afterdischarge.

METHODS

Abdominal ganglia were dissected from sexually mature *Aplysia californica* (300–800 g) anesthetized by the injection of isotonic $MgCl_2$ (approximately one-third of the body volume) into the hemocoel. For each experimental trial, an abdominal ganglion was pinned dorsal surface up onto a clear resin (Sylgard) dish, and a bipolar stimulating electrode was placed on the larger of the two bag-cell clusters. A single bag cell adjacent to the stimulating electrode was penetrated with a glass microelectrode filled with 2 *M* potassium acetate to monitor activity in the electrically coupled bag-cell cluster. Bag-cell afterdischarge was initiated by a brief train of current passed through the stimulating electrode.

During dissection and preparation of the ganglion, 50 μCi of [γ-^{32}P]ATP (New England Nuclear; 2900 Ci/mmol) was lyophylized in a 0.5-ml microcentrifuge tube using a Speed Vac concentrator (Savant). The lyophilisate was redissolved in approximately 50 nl of 300 m*M* potassium phosphate buffer (pH 6.8) and loaded into polyethylene tubing (PE-10) for transfer to a glass microelectrode. Microelectrodes

were prepared for each experiment by pulling glass tubing (Kwik-Fil, W.P. Instruments) and mechanically breaking the tip to obtain a diameter suitable for low-pressure (0.5–5 kg/cm^2) injection of the ATP solution. The labeled ATP in phosphate buffer was transferred to the blunt end of the microelectrode and drawn into the tip by capillary action. The microelectrode was then assembled into a holder suitable for pressure injection (W.P. Instruments).

In each control and experimental trial, a single neuron (L6 or R15) was pressure injected with approximately 5 nl of the [^{32}P]ATP over a 5- to 15-min period. Control ganglia were frozen in liquid nitrogen 60–75 min after the beginning of ATP injection without further treatment. Experimental ganglia were treated by either of two methods. In order to affect the long-term activity of L6 and R15 by a "natural" stimulus, we evoked afterdischarge of the bag cells using brief trains of current pulses applied through a bipolar electrode. Pharmacological excitation of L6 was accomplished by bathing the ganglion in 300 μM IBMX dissolved in artificial seawater (ASW). Each experiment was terminated by freezing the ganglion in liquid N_2 at the peak response to treatment. We defined the peak response as the maximal inhibition and maximal burst augmentation of L6 and R15, respectively. Electrophysiological responses for each of the conditions are represented in the traces shown in Fig. 1. Regardless of the treatment, all ganglia were frogen in liquid nitrogen approximately 60–75 min after the beginning of isotope injection to assure equivalent periods of isotope incorporation.

Proteins were extracted from individual ganglia using methods similar to those of Lemos *et al.* (1982). Each ganglion was homogenized in 400 μl of stop buffer (50 m*M* MOPS, pH 7.0, 20 m*M* EDTA, 10 m*M* NaF, 1 m*M* ATP) at 4°C. The connective tissue sheath was removed and the proteins were precipitated by the addition of 20% trichloroacetic acid (TCA) at 4°C. The precipitate was washed twice with 10% TCA and twice with chloroform/methanol [1:2, then 2:1 (v:v)] to extract the phospholipids. The proteins were redissolved in 40 μl of sodium dodecyl sulfate (SDS) sample buffer (Laemmli, 1970), boiled for 2 min, and resolved by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (0.75 mm × 20 cm; 8–15% gradient; 150 V, 18 hr) (O'Farrell *et al.*, 1977). Molecular weight standards (Sigma) were coelectrophoresed with the extracted proteins. The phosphorylated proteins were visualized by autoradiography with Kodak X-Omat AR X-ray film using Cronex intensifying screens (Dow) at −70°C. To quantify label incorporation into protein, the autoradiograms were scanned using a Joyce–Loebl microdensitometer and peak areas were computed using a Zeiss MOP-3 scanner.

To normalize the relative labeling of phosphoproteins from different experiments, the peak areas between 32,000 and 45,000 daltons and between 50,000 and 66,000 daltons were summed as the standard combined peak area (SCPA), and the ratio of specific peak areas to SCPA was computed. The peak areas in these molecular weight ranges were chosen as the standard for normalization since they represented a major portion of the phosphoproteins present and were not significantly affected by our experimental treatments. The validity of using the SCPA as a normalizing value was tested by comparing the normalized peak value for another major group of phosphoproteins. As indicated in Table I, there was no significant change in the normalized peak areas for phosphoproteins of between 17,000 and 23,000 daltons under any of the control or experimental conditions for either L6 or R15. This

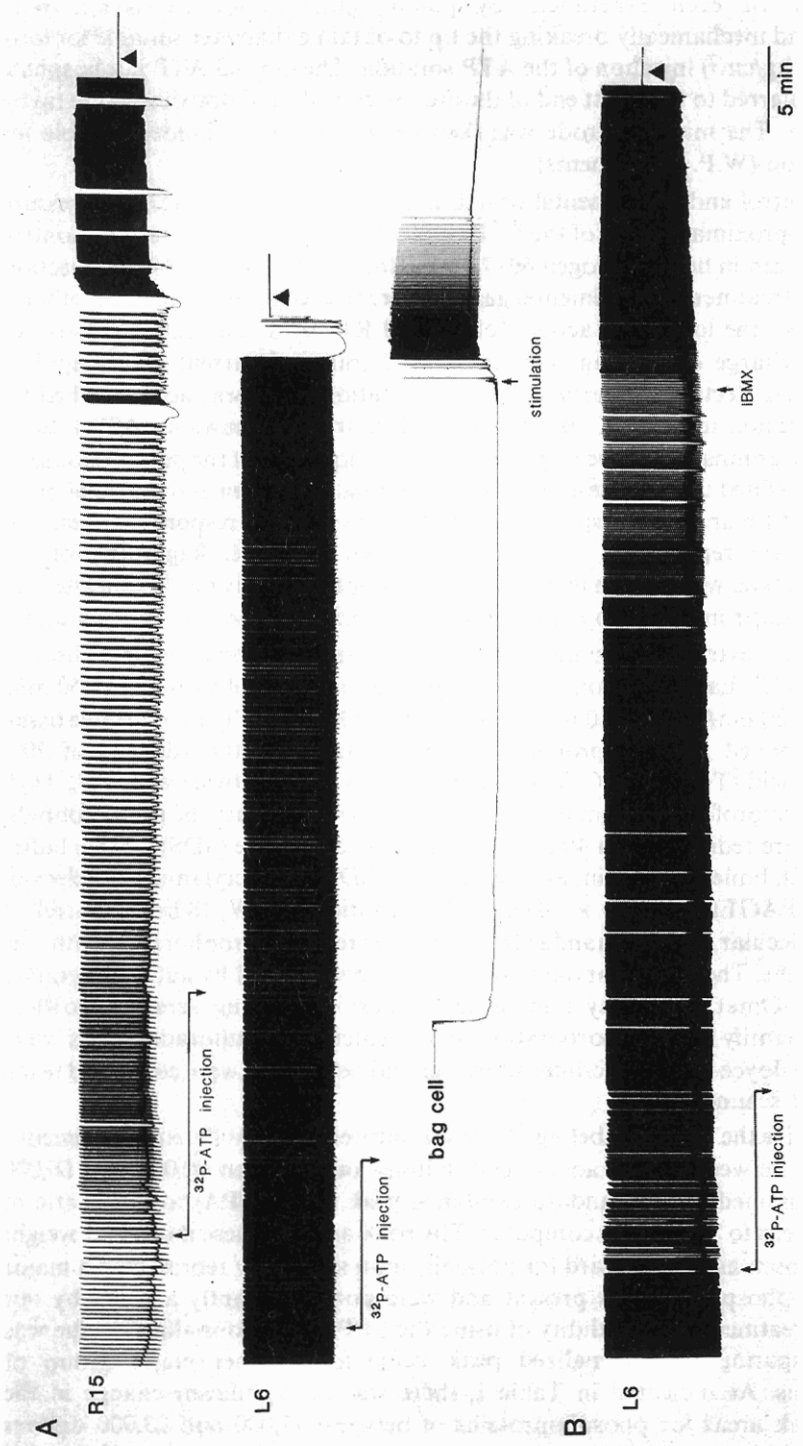

Fig. 1. Natural and pharmacological treatments affecting long-term activities of neurons R15 and L6. (A) Composite of intracellular recordings from two experiments showing the excitatory response of R15 (upper trace) and the inhibitory response of L6 (middle trace) to an electrically triggered discharge of bag-cell activity. The lower trace shows a representative bag-cell afterdischarge (BCA) induced by brief electrical stimulation (arrow). The excitatory (R15) and inhibitory (L6) responses to BCA were allowed to develop to maximal levels before the microelectrode was removed (end of recording) and the ganglion frozen in liquid nitrogen (triangle). (B) Long-term excitation of L6 following the bath application of 300 μM isobutylmethylxanthine (IBMX). Excitatory responses of L6 to IBMX developed over a 10- to 20-min period and the ganglia were frozen (triangle) at the peak response.

Table I. (A) Normalized Mean Peak Areas for the 17,000- to 23,000-, 29,000-, and 150,000-Dalton Phosphoproteins in Neurons L6 and R15 and (B) Significance Levels for Comparisons of Means for Phosphoproteins in Neurons L6 and R15

A

Treatment	*N*	17–23 kD Mean	17–23 kD (SD)	29 kD Mean	29 kD (SD)	150 kD Mean	150 kD (SD)
L6 control	4	0.26	(0.11)	0.20	(0.07)	0.10	(0.08)
L6 + BCA	4	0.31	(0.18)	0.09	(0.11)	0.03	(0.02)
L6 + IBMX	5	0.31	(0.24)	0.39	(0.16)	0.11	(0.07)
R15 control	6	0.25	(0.18)	0.16	(0.07)	0.04	(0.03)
R15 + BCA	4	0.31	(0.10)	0.13	(0.04)	0.07	(0.04)

B

Treatment	L6 + BCA	L6 + IBMX	R15 control	R15 + BCA
17–23 kD				
L6 control	0.5	0.5	0.5	—
L6 + BCA	—	0.5	—	—
R15 control	—	—	—	0.5
29 kD				
L6 control	0.3	0.02	0.4	—
L6 + BCA	—	0.01	—	—
R15 control	—	—	—	0.4
150 kD				
L6 control	0.1	0.5	0.1	—
L6 + BCA	—	0.05	—	—
R15 control	—	—	—	0.2

demonstrated that the distribution of phosphorylation between the SCPA and other phosphoproteins was consistent for all trials and that the SCPA could be used as an internal standard. The normalized peak areas for individual phosphoproteins from control and experimental trials were compared using a *t* test for two means.

RESULTS

The pressure injection of [γ-^{32}P]ATP into L6 and R15 had no apparent long-term effect on the spontaneous activity of the cells, or upon their responses to bag-cell afterdischarge. As shown in Fig. 1, the mechanical disturbance associated with injection of the isotope usually caused a slight depolarization of the cells, but recovery normally occurred within 5–20 min, after which the cells displayed normal patterns of activity. If normal electrical activity of a neuron did not resume following isotope injection, the trial was terminated.

Following a postinjection incubation period of 40 to 60 min, the activity of L6 or R15 was altered in experimental trials either by eliciting bag-cell afterdischarge or by bath applying IBMX (L6 experiments only). As shown in Fig. 1A, the amplitude and time course of bag cell-induced inhibition of L6 and excitation (burst augmentation) of R15 were normal. Likewise, the excitatory response of L6 (Fig. 1B) to treatment with

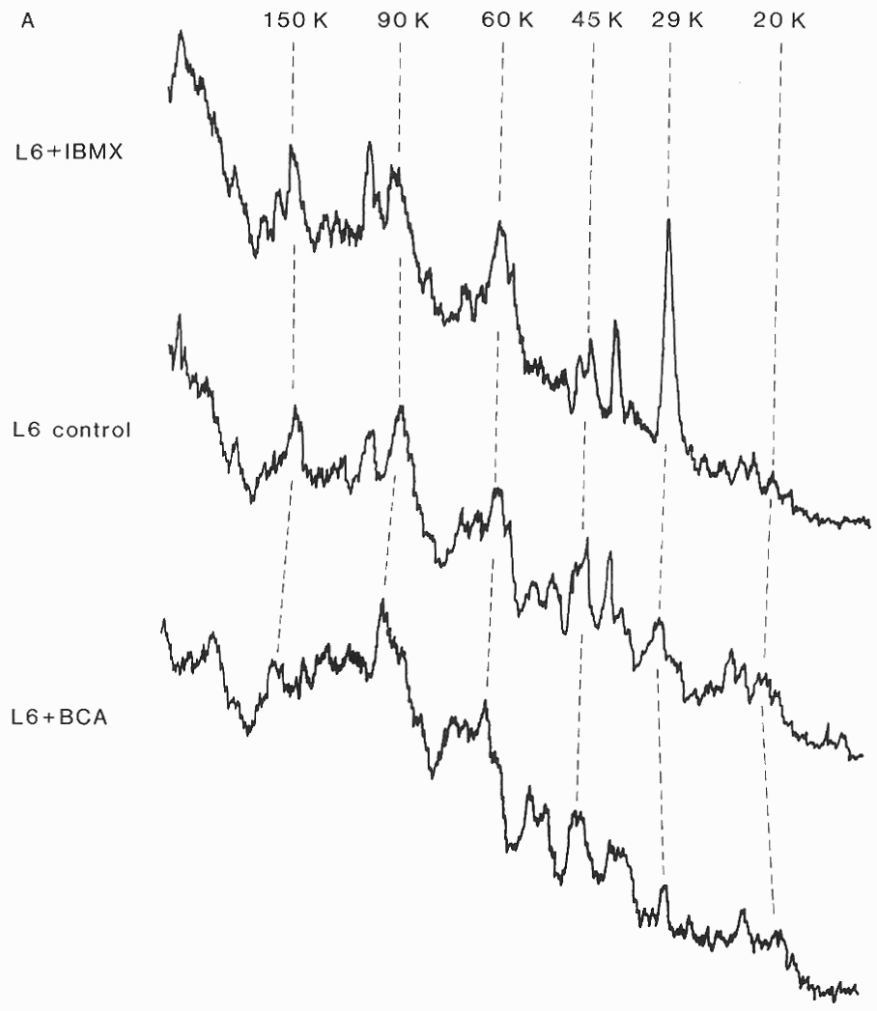

Fig. 2. (A) Densitometric scans of representative autoradiograms of phosphorylated proteins in neuron L6. Protein phosphorylation in neuron L6 under control conditions is compared with phosphorylation following BCA and IBMX treatments. The apparent decrease in phosphorylation of the 29,000-dalton protein following BCA and increase following IBMX application are evident. (B) Densitometric scans of representative autoradiograms of phosphorylated proteins in neuron R15. Protein phosphorylation in neuron R15 under control conditions (normal bursting) and following bag-cell afterdischarge (BCA) (burst augmentation) is compared with that of neuron L6 under control conditions (normal bursting). The similar patterns of protein phosphorylation in R15 under the two conditions and the similar patterns in R15 and L6 under control conditions are evident.

IBMX was unaffected by the injection procedure. From these observations we conclude that the intracellular injection procedure and the slight increase in volume of the soma associated with isotope injection had no long-term effect on the resting membrane conductance or the response mechanisms of the cells.

The patterns of protein phosphorylation associated with baseline levels of activity in neurons L6 and R15 (Figs. 2A and B) were similar. Furthermore, despite differences

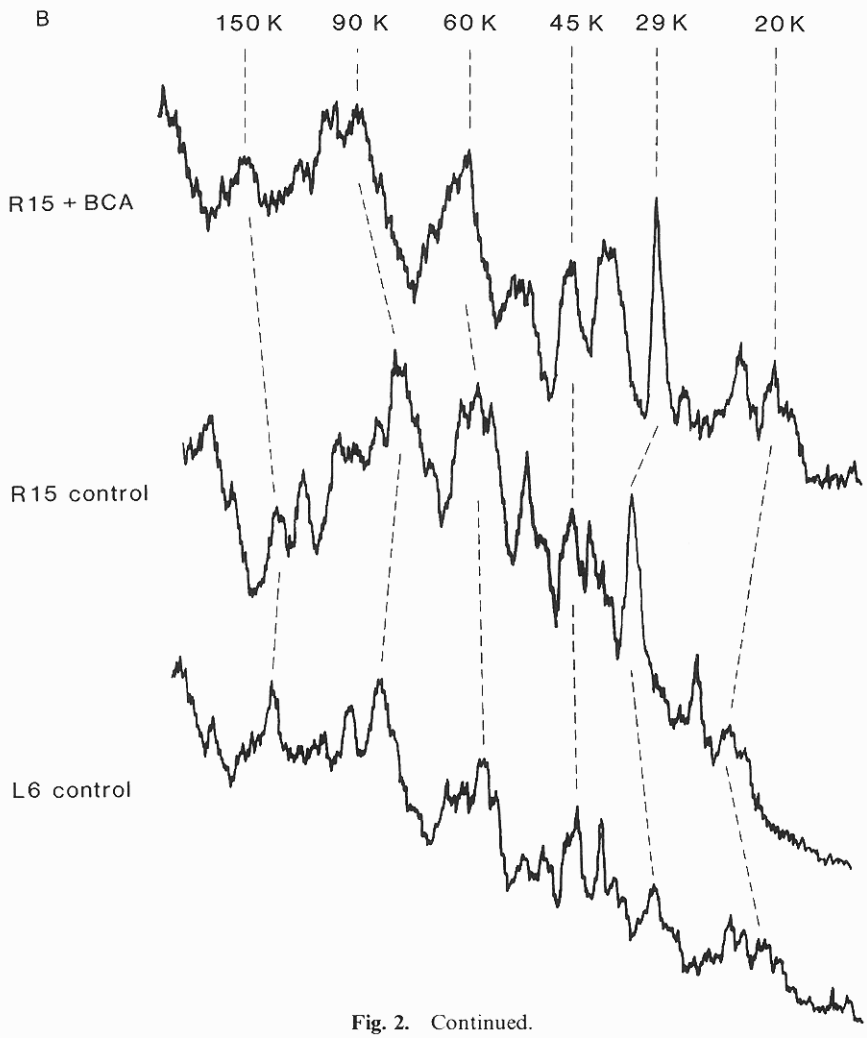

Fig. 2. Continued.

in their physiological properties (Frazier *et al.*, 1967) and developmental origins (Kriegstein, 1977), the levels of phosphorylation of proteins in the two neurons were not significantly different (Table I).

Only neuron L6 exhibited a consistently detectable change in protein phosphorylation during inhibition by bag-cell afterdischarge. Although most of the 15 phosphoproteins remained unchanged (Fig. 2A), there was an apparent twofold decrease in the level of phosphorylation of a 29,000-dalton (29-kD) protein compared with controls (Table IA). However, the observed effect of bag-cell activity did not represent a significant change compared with controls (Table IB). The lack of statistical significance of this value is due to a large extent to the increased variance introduced by one trial which fell within control levels of phosphorylation. If this trial was omitted, the change in phosphorylation of the 29,000-dalton protein would be statistically significant ($P < 0.02$). A 150,000-dalton protein (150 kD) also appeared to undergo

dephosphorylation during inhibition of L6, but the change was not statistically significant either.

The observation that the 29- and 150-kD proteins might be dephosphorylated during inhibition of L6 led us to apply a phosphodiesterase inhibitor, IBMX, to the neuron in order to compare the levels of phosphorylation of these proteins and others during electrical excitation. The bath application of IBMX strongly excited L6, as it does other neurons such as R15 (Levitan and Norman, 1980). As indicated in Table IA, IBMX treatment of L6 resulted in a twofold increase in the level of phosphorylation of the 29-kD protein relative to controls. The increase was statistically significant ($P < 0.02$) (Table IB). There was no observed change in the level of phosphorylation of the 150-kD protein or other phosphoproteins in the cell. Thus, the level of phosphorylation of the 29-kD protein correlates with the impulse activity of the neuron. The difference in mean phosphorylation of this protein during excitation induced by IBMX and during inhibition induced by bag-cell afterdischarge was highly significant ($P < 0.01$).

Burst augmentation of R15 induced by bag-cell afterdischarge resulted in no apparent change in the general pattern of phosphorylation (Fig. 2B) or the levels of phosphorylation of either the 29- or the 150-kD protein (Table I). We did not examine the effect of IBMX on protein phosphorylation in R15.

DISCUSSION

Previous studies of protein phosphorylation in *Aplysia* neurons have relied on the intracellular injection of protein kinases and/or the bath or direct application of neurotransmitters. Using these techniques it is difficult to determine the extent to which the treatment is spatially and quantitatively duplicating natural physiological events. Therefore, treatments may result in greater or lesser changes in protein phosphorylation than those occurring *in vivo*. To circumvent this problem, we examined changes in protein phosphorylation induced by the endogenous release of transmitters from the peptidergic bag-cell neurons associated with the *Aplysia* abdominal ganglion.

Relatively small changes in the extent of phosphorylation of a particular protein may mediate a physiological response under natural conditions. Our experiments suggest that it may be useful and perhaps necessary in some cases to enhance the difference between two states of neuronal activity to detect small but physiologically important changes in protein phosphorylation. In neuron L6, the mean phosphorylation of a 29-kD protein decreased approximately twofold during the inhibition induced by bag-cell afterdischarge (BCA). The mean phosphorylation of this protein increased approximately twofold during the excitation induced by bath application of the phosphodiesterase inhibitor IBMX. The difference between the IBMX mean and the control mean was statistically significant ($P < 0.02$). The difference between the bag-cell afterdischarge mean and the control mean was not ($P < 0.3$). However, the difference would be statistically significant if a single trial were omitted. In addition, the difference in mean phosphorylation of the 29-kD protein in the two most different states of electrical activity, excitation induced by IBMX and inhibition induced by BCA, was highly significant ($P < 0.01$).

We did not detect statistically significant changes in the phosphorylation of proteins in neuron R15 during burst augmentation induced by bag-cell afterdischarge. This does not preclude the possibility that protein phosphorylation in this neuron is altered by afterdischarge. For example, phosphoproteins comigrating with more densely labeled proteins or proteins present in small quantities would not be detected. Also, we might have detected changes if the experiments had been terminated at some other time during the response or if we had utilized a second treatment which would have brought about long-term inhibition of the cell. This would have allowed a comparison of protein phosphorylation at two more clearly defined states of activity.

Our data suggest that phosphorylation of most major proteins in *Aplysia* neurons L6 and R15 is not significantly changed by treatments that alter the electrical activity of these cells. This observation is consistent with the view that most phosphoproteins in neurons function in a wide range of metabolic pathways (reviewed by Cohen, 1982) and that the extent of phosphorylation of these proteins is not directly related to the state of electrical activity.

With respect to the 29-kD phosphoprotein in neuron L6, our data are consistent with the hypothesis that cyclic nucleotide-dependent protein phosphorylation may be responsible for some changes in the electrical activity of neurons (Kuo and Greengard, 1978; Greengard, 1978). Other investigators have provided evidence that protein kinases regulate the activity of molluscan neurons by phosphorylation of specific protein substrates (reviewed by Levitan *et al.*, 1984). Recent examination of the mechanism by which this occurs in several *Aplysia* neurons suggests that phosphorylation of specific protein substrates by cAMP-dependent protein kinases may modulate K^+ conductances. In our study, application of the phosphodiesterase inhibitor IBMX led to long-term excitation of neuron L6. Previous investigations of *Aplysia* neurons have shown that IBMX treatment results in increased cyclic nucleotide concentrations (Levitan and Norman, 1980) and stimulates protein phosphorylation (Paris *et al.*, 1981). We were also able to manipulate the activity of neuron L6 by stimulating afterdischarge of the peptidergic bag cells. The resulting inhibition is due in part to a large, prolonged increased K^+ conductance (Brownell and Mayeri, 1979). It is possible that the IBMX-induced excitation of L6 is due in part to a decreased K^+ conductance; however, this remains to be demonstrated. If this is the case, the two opposite responses of neuron L6 to the two treatments may correspond to different states of K^+ conductance, which would in turn correlate with the level of phosphorylation of the 29-kD protein.

Lemos *et al.* (1984) showed that synaptic stimulation alters protein phosphorylation in neuron R15. The resulting long-lasting hyperpolarization is due to at least two conductance changes which correlate with changes in phosphorylation of several proteins including the increased phosphorylation of a 29-kD species. The effect on the 29-kD protein is mimicked by the application of serotonin. More recently, Lemos *et al.* (1985) reported that two phosphoproteins, 29 and 70 kD, cannot be pharmacological and kinetically dissociated from the serotonin-induced K^+ conductance increase in R15, suggesting that these species are closely associated with, and perhaps responsible for, the conductance change.

Goy *et al.* (1984) recently identified a 29-kD protein in a lobster neuromuscular preparation that is phosphorylated following treatment with serotonin. As in our

study, the 29-kD protein is phosphorylated by the application of the phosphodiesterase inhibitor IBMX at approximately the same concentration. Goy *et al.* provide substantial evidence that phosphorylation of the protein is mediated by a cyclic nucleotide-dependent mechanism. Whether or not our results and those of Lemos *et al.* and Goy *et al.* are related with respect to the identified 29-kD proteins requires further study.

ACKNOWLEDGMENTS

We thank A. Millemann for her technical assistance and M. Kirk and M. Goy for critically reading portions of the manuscript. This work was supported in NIH Grant NS18681 (PHB) and an Oregon Medical Research Foundation Grant (PDS).

REFERENCES

Branton, W. D. (1978). Evidence for mediation of a neuronal interaction by a behaviorally active peptide. *Proc. Natl. Acad. Sci. USA* **75:**5732–5736.

Brownell, P., and Mayeri, E. (1979). Prolonged inhibition of neurons by neuroendocrine bag cells. *Science* **204:**417–420.

Cohen, P. (1982). The role of protein phosphorylation in neural and hormonal control of cellular activity. *Nature* **296:**613–620.

Frazier, W. T., Kandel, E. R., Kupfermann, I., Waziri, R., and Coggeshall, R. E. (1967). Morphological and functional properties of identified neurons in the abdominal ganglion of *Aplysia californica*. *J. Neurophysiol.* **30:**1288–1351.

Goy, M. F., Schwarz, T. L., and Kravitz, E. A. (1984). Serotonin-induced protein phosphorylation in a lobster neuromuscular preparation. *J. Neurosci.* **4:**611–626.

Greengard, P. (1978). Phosphorylated proteins as physiological effectors. *Science* **199:**146–152.

Kennedy, M. B. (1983). Experimental approaches to understanding the role of protein phosphorylation in the regulation of neuronal function. *Annu. Rev. Neurosci.* **6:**493–425.

Kriegstein, A. R. (1977). Development of the nervous system of *Aplysia californica*. *Proc. Natl. Acad. Sci. USA* **74:**375–378.

Kuo, J., and Greengard, P. (1969). Cyclic nucleotide-dependent protein kinase, IV. Widespread occurrence of adenosine 3′5′ monophosphate-dependent protein kinase in various tissues and phyla of the animal kingdom. *Proc. Natl. Acad. Sci. USA* **64:**1349–1355.

Kupfermann, I. (1980). Role of cyclic nucleotides in excitable cells. *Annu. Rev. Physiol.* **42:**629–649.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:**680–685.

Lemos, J., Novak-Hofer, I., and Levitan, I. (1982). Serotonin alters the phosphorylation of specific proteins inside a single living nerve cell. *Nature* **298:**64–65.

Lemos, J. R., Novak-Hofer, I., and Levitan, I. B. (1984). Synaptic stimulation alters protein phosphorylation *in vivo* in a single *Aplysia* neuron. *Proc. Natl. Acad. Sci. USA* **81:**3233–3237.

Lemos, J. R., Novak-Hofer, I., and Levitan, I. B. (1985). Phosphorproteins associated with the regulation of a specific potassium channel in the identified *Aplysia* neuron R15. *J. Biol. Chem.* **260:**3207–3214.

Levitan, I. B., and Adams, W. B. (1981). Cyclic AMP modulation of a specific ion channel in an identified nerve cell: Possible role for protein phosphorylation. *Adv. Cyclic Nucleotide Res.* **14:**647–653.

Levitan, I. B., and Norman, J. (1980). Different affects of cAMP and cGMP derivatives on the activity of an identified neuron: Biochemical and electrophysiological analysis. *Brain Res.* **187:**415–429.

Levitan, I. B., Lemos, J. R., and Novak-Hofer, I. (1984). Protein phosphorylation and the regulation of ion channels. *Trends Neurosci.* **6:**496–499.

Nestler, E. J., and Greengard, P. (1983). Protein phosphorylation in the brain. *Nature* **305:**583–588.

Nestler, E. J., Walaas, S. I., and Greengard, P. (1984). Neuronal phosphoproteins: Physiological and clinical applications. *Science* **225:**1357–1364.

O'Farrell, P., Goodman, H., and O'Farrell, P. (1977). High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12:**1133–1142.

Paris, C. G., Castellucci, V. F., Kandel, E. R., and Schwartz, J. H. (1981). Protein phosphorylation, presynaptic facilitation, and behavioral sensitization in *Aplysia*. *Cold Spring Harb. Conf. Cell Prolif.* **8:** 1361–1375.

Rothman, B. S., Mayeri, E., Brown, R. O., Yuan, P.- M., and Shively, J. E. (1983). Primary structure and neuronal effects of α-bag cell peptide, a second candidate neurotransmitter encoded by a single gene in a bag cell neurons of *Aplysia*. *Proc. Natl. Acad. Sci. USA* **80:**5753–5757.

Siegelbaum, S. A., and Tsien, R. W. (1983). Modulation of gated ion channels as a mode of transmitter action. *Trends Neurosci.* **6:**307–313.