Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 2036–2039, March 1990
Neurobiology

Sensitizing stimuli cause translocation of protein kinase C in *Aplysia* sensory neurons

(sensitization/presynaptic facilitation/serotonin/Mg^{2+}/learning and memory)

TODD C. SACKTOR AND JAMES H. SCHWARTZ

Howard Hughes Medical Institute, Department of Neurology, Center for Neurobiology and Behavior, College of Physicians & Surgeons, Columbia University, 722 West 168th Street, New York, NY 10032

Communicated by Ora M. Rosen, December 15, 1989 (received for review July 17, 1989)

ABSTRACT **The defensive tail-withdrawal reflex of *Aplysia californica*, mediated by identified sensory neurons in pleural ganglia that form synapses on motor cells in pedal ganglia, can be sensitized by stimulating the animal with electric shock. The neurophysiological basis of this simple form of learning is thought to be the increased release of transmitter by the sensory neurons. Earlier work has focused on cAMP-dependent protein phosphorylation as the cause of the presynaptic facilitation underlying short-term sensitization. Using physiological concentrations of Mg^{2+} during fractionation, we now find that, independent from cAMP, protein kinase C is translocated in sensory neurons by sensitizing stimuli. Translocation occurred after behavioral training of the animal and after application to isolated ganglia of serotonin or phorbol esters. Taken together with the neurophysiological evidence presented in the accompanying paper that phorbol esters can produce the facilitation, these biochemical results suggest that protein kinase C plays a role in producing the presynaptic facilitation that underlies short-term sensitization and dishabituation of defensive reflexes.**

Increased release of transmitter from abdominal or pleural sensory neurons (presynaptic facilitation) is a cellular mechanism underlying behavioral sensitization of withdrawal reflexes in *Aplysia* (1, 2). It has been proposed that the presynaptic facilitation is caused by closure of a serotonin-sensitive potassium (K_S) channel through cAMP-dependent protein phosphorylation that results in prolonging the action potential in terminals of the sensory neurons (3, 4). Closure of the K_S channel does not facilitate depressed sensory-to-motor synapses, however, suggesting that some other mechanism might also contribute to the facilitation (5, 6). We now show that sensitizing stimulation of the animals translocates and activates the Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C, PKC) (7).

MATERIALS AND METHODS

Aplysia. *Aplysia* (150–250 g from the Howard Hughes Medical Institute Mariculture Resource Facility, Woods Hole Oceanographic Institution, MA, or Sea Life Supply, Sand City, CA) were isolated in individual cages 1 week before testing.

Training. The protocol was modified from Scholz and Byrne (8): 10 12.5-mA shocks repeated four times at 0.5-hr intervals were delivered to one side of the animal, resulting in ipsilateral sensitization. After the last train of shocks, animals were anesthetized first by immersion in isotonic $MgCl_2$/unsupplemented artificial seawater, 1:1 (vol/vol), followed by injection of $MgCl_2$ (9). Pairs of pleural–pedal ganglia were isolated in an artificial seawater containing high Mg^{2+} (220 mM) and immediately were homogenized at 4°C in 0.3 ml of homogenization buffer (50 mM Tris·HCl, pH 7.5/1 mM EGTA/10 mM $MgCl_2$/5 mM 2-mercaptoethanol/0.1 mM phenylmethylsulfonyl fluoride/50 kallikrein units of aprotinin per ml/5 mM benzamidine/0.1 mM leupeptin). (Unless otherwise specified, chemicals were from Sigma.) The homogenate was centrifuged at 1000 × *g*; the resulting supernatant was centrifuged again at 100,000 × *g* for 0.5 hr to obtain a supernatant (cytosol) and a pellet. This membrane fraction was resuspended in 0.3 ml of homogenization buffer.

Serotonin (5-HT) and cAMP analog treatment. Symmetric pairs of pleural–pedal ganglia, dissected from a single animal as described above, were washed twice more with normal (55 mM Mg^{2+}) supplemented seawater. One of the pleural–pedal ganglia was exposed to seawater containing 20 μM 5-HT for 5 min or to seawater containing 1 mM 8-(4-chlorophenylthio)-cAMP for 45 min, and the contralateral (control) ganglia were exposed to seawater alone. The washes were then replaced with the buffer at 4°C, and neuronal components (cell bodies and neuropil) were dissected and fractionated as described above.

Phorbol ester and 4α-phorbol treatment. Neuronal components were dissected from paired pleural–pedal ganglia in the high-Mg^{2+} seawater and transferred to glass–glass tissue grinders containing 0.2 ml of normal seawater. The seawater was replaced twice at 5-min intervals. One of the pair of ganglia was exposed to seawater containing 0.2 μM phorbol 12,13-dibutyrate or 4α-phorbol for 45 min at 15°C, and the contralateral ganglia were exposed to seawater alone. Experimental solutions were replaced with 0.3 ml of homogenization buffer at 4°C and then homogenized and centrifuged as above.

Sensory clusters. Pairs of pleural–pedal ganglia from five anaesthetized *Aplysia* were isolated, the right and left alternately pinned separately in the high-Mg^{2+} seawater. After two 5-min washes with normal seawater and a 5-min incubation with either seawater containing 20 μM 5-HT or normal seawater, sensory cell clusters were dissected out in propylene glycol/2 M NaCl, 1:1 (vol/vol), at −20°C (10) and were homogenized at 4°C in 0.2 ml of the buffer. After high-speed centrifugation, the supernatant was removed, and the pellet was resuspended in 0.2 ml of the buffer.

Assays for PKC. *Histone phosphorylation.* Cytosol and membrane fractions were diluted 1:4 (ganglia) or 1:1 (sensory cell clusters) with the buffer containing Triton X-100 (0.05% final detergent concentration). At this dilution, which contains <0.3 μg of protein in a 10-μl sample, the effect of endogenous inhibitors on histone phosphorylation is negligible, circumventing the need to purify the kinase further. At twice this concentration of protein, phosphorylation of histone plateaus at 30 min, but under our conditions, the reaction proceeded at a constant rate for 1 hr (data not shown). The reaction mixture (30 μl) contained 50 mM

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PKC, protein kinase C; 5-HT, serotonin.

Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mg of cAMP-dependent protein kinase (PKA) inhibitor (type II) per ml, and 0.6 mg of histone H1 (III-S) per ml with or without synthetic dioleoyl phosphatidylserine (Avanti Polar Lipids) at 150 μg/ml and 200 nM phorbol 12-myristate 13-acetate. After addition of 10 μl of the *Aplysia* sample, the reaction was started with 10 μl of [^{32}P]ATP (New England Nuclear; 1 μCi per tube; 1 Ci = 37 GBq), 50 μg final concentration. After 0.5 hr at 20°C, 40 μl were spotted onto Whatman phosphocellulose paper, then washed six times for 5 min with 0.425% phosphoric acid, and assayed by scintillation counting. PKC activity was measured in triplicate as the difference in histone phosphorylation with and without phosphatidylserine and the phorbol ester. Because these cofactors stimulate all of the isozymes of PKC even in the absence of Ca^{2+} (see refs. 11 and 12), we assayed the kinase in the presence of EGTA (final concentration, 0.2 mM) to minimize the contribution of the Ca^{2+}/calmodulin-dependent protein kinase and any effects of Ca^{2+}-dependent proteases.

Binding of [^{3}H]phorbol 12,13-dibutyrate. Phorbol ester binding, performed in duplicate, was measured as described by Uchida and Filburn (13) with modifications. Samples (20 μl) of undiluted cytosol or membrane fractions were added to 0.4 ml of 25 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/1.4 mM $CaCl_2$/0.4 mM EDTA/4 mg of bovine serum albumin (Calbiochem) per ml/0.02% Na azide/100 μg of phosphatidylserine per ml/5 nM [20-^{3}H]phorbol 12,13-dibutyrate (New England Nuclear; 40 nCi per tube) with or without 1 μM unlabeled phorbol dibutyrate. After 2 hr at 4°C, the reaction was stopped by filtration through Whatman GF/F disks, then rinsed three times with 5 ml of 20 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/1 mM $CaCl_2$ at 4°C. The filters were dried and counted by scintillation. Specific binding is the difference between binding with and without excess unlabeled phorbol dibutyrate.

Protein in fractions from the total neural components of pleural–pedal ganglia was measured by the Bradford microassay (Bio-Rad) as modified by Simpson and Sonne (14). Protein in fractions from sensory clusters was determined in 50-μl samples of supernatant or 100 μl of resuspended pellet by adding 0.1 M NaOH (50 μl) followed by dye reagent no. 1 of Read and Northcote (15) with bovine serum albumin as standard.

RESULTS

Effect of Mg^{2+} on the Subcellular Distribution of PKC in Neuronal Tissue. Ca^{2+} present during homogenization has been shown to affect the subcellular distribution of PKC, translocating the enzyme to membrane (16). This redistribution is thought to reflect the physiological activation of the enzyme when intracellular Ca^{2+} is increased (17). We asked if Mg^{2+} might also have an effect. Mg^{2+} causes a fraction of PKC to dissociate from the membrane (Fig. 1). Homogenization in a buffer with concentrations of Mg^{2+} that are physiological for marine mollusks (10 mM; see ref. 18) and no Ca^{2+} (1 mM EGTA) results in a lower basal activity on the membrane, without subsequently affecting the ability of either Ca^{2+} or phorbol esters to translocate PKC or diminishing the total activity of the enzyme (data not shown). Because these conditions might reflect the actual state of the enzyme within resting neurons, we homogenized the tissue in 10 mM Mg^{2+} to study the effect of sensitizing stimuli on the translocation of PKC.

Translocation of PKC by Sensitizing Stimuli. Mild electrical stimulation of one side of the animal produces both short-term and long-term sensitization of withdrawal reflexes only on that side (8). Because pleural sensory cells are thought to synapse within the neuropil of the ipsilateral pleural and pedal ganglia (19), we first measured the activity of PKC on the membrane of the ganglia dissected from the stimulated side and used the contralateral (unstimulated) ganglia as controls. Assaying within 5 min after the training, we found that the activity of PKC associated with membrane increased by 37 ± 15% (Fig. 2, training column; mean ± SEM, $P < 0.05$; this and subsequent P values were determined by paired t tests). No change in the amount of enzyme activity in the cytosol was detectable (3.9 ± 4%), perhaps because the amounts of PKC in these experiments are several-fold higher in cytosol (1065 ± 57 pmol/min per mg) than membrane (200 ± 28).

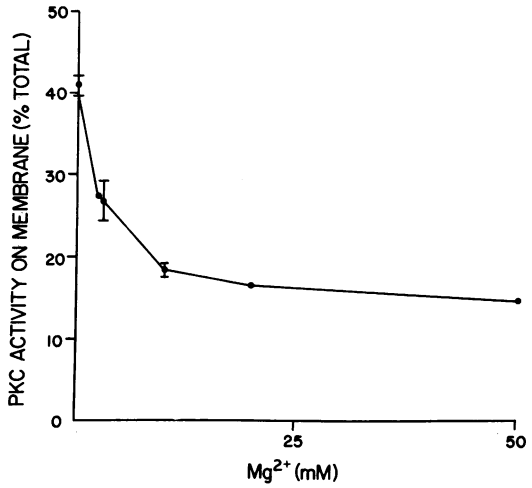

FIG. 1. Effect of Mg^{2+} on the subcellular distribution of *Aplysia* neuronal PKC. After isolation of pairs of pleural–pedal ganglia as described, the high-Mg^{2+} seawater was replaced by 0.8 M sucrose (pH 8) for 5 min. Samples (200 μl) of the 1000 × g supernatant from the homogenate were then prepared as described, and Mg^{2+} was added in the final concentrations indicated. Samples with no added Mg^{2+} contained 1.0 mM EDTA. After centrifugation at 100,000 × g for 1 hr, PKC activity was assayed by histone phosphorylation. Error bars indicate SEM (n = 4). At 10 mM Mg^{2+}, the total activity in the sample was 5834 ± 854 pmol/min. The supernatant contained 31.5 ± 1.3 μg of protein; the pellet contained 21.2 ± 1.4 μg of protein.

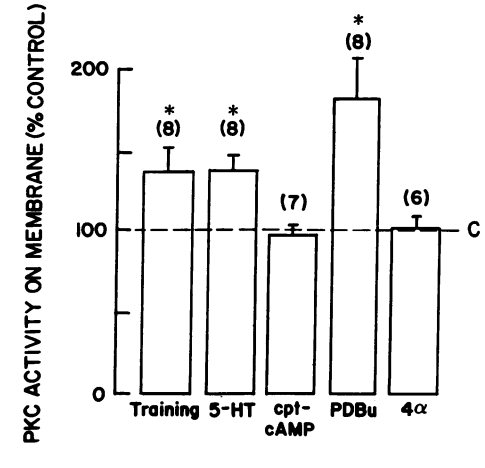

FIG. 2. Translocation of PKC activity to membrane. Training and treatments of isolated pairs of ganglia with both 20 μM 5-HT for 5 min and 0.2 μM phorbol dibutyrate (PDBu) for 45 min result in translocation of the kinase. Treatments with inactive 4α-phorbol at 0.2 μM for 45 min and with a permeating analog of cAMP, 8-(4-chlorophenylthio-cAMP (cpt-cAMP), at 1 mM for 45 min were not effective. The number of experiments is given in parentheses; an asterisk indicates significance (paired t test). Amounts of PKC on membrane in experimental ganglia relative to control ganglia are presented as the mean percentage ± SEM of the specific activity on control membranes (C; mean specific activity of the kinase on membrane was 436 ± 31 pmol/min per mg; n = 37).

To examine the molecular events that trigger this increase of membrane-associated kinase, we next dissected pairs of intact pleural–pedal ganglia. 5-HT, a transmitter presumed to be released during electrical stimulation (1), causes facilitation of sensory-to-motor cell synapses and increases cAMP in sensory cells (3, 4, 10, 20, 21). Application of 20 μM 5-HT for 5 min to the isolated ganglia, a concentration shown to result in maximal facilitation (M. Klein, personal communication), resulted in translocation of PKC to membrane as assayed both by histone phosphorylation (Fig. 2, 5-HT column; 38 ± 11%, $P < 0.02$) and by binding of [^{3}H]phorbol dibutyrate (20 ± 7%, $P < 0.05$; Fig. 3). We used the phorbol dibutyrate-binding assay as another method to show that histone phosphorylation truly measures PKC. PKC in cytosol as determined by histone phosphorylation decreased by 5.7 ± 3%.

To show that facilitory stimuli do not act on PKC through cAMP, we applied an effective (9) membrane-permeable analog, 8-(4-chlorophenylthio)-cAMP, at 1 mM for 45 min to the isolated ganglia. No translocation of the kinase to membrane (−1.7 ± 6%) was observed (Fig. 2, *cpt-cAMP* column). The analog at 0.1 mM together with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine at 0.1 mM for 2 hr, a condition that greatly enhances cAMP-dependent phosphorylation in *Aplysia* neuronal tissue (22), also did not translocate PKC to membrane (4.7 ± 6%). The effect of 5-HT on PKC may be produced by a serotonin receptor different from the one that activates adenylyl cyclase.

Phorbol esters are pharmacological agents that circumvent the receptor-mediated generation of endogenous lipid second messengers by activating PKC directly (7, 23). Exposure of ganglia to 0.2 μM phorbol dibutyrate for 45 min resulted in an 81 ± 26% increase in membrane-bound PKC (Fig. 2, PDBu column) and a loss of 10 ± 5% from the cytosol. The inactive 4α-phorbol was ineffective (1.2 ± 8.8%; Fig. 2, 4α column). None of the treatments resulted in the loss of total PKC activity.

Since both electrical stimulation and application of 5-HT enhance release of transmitter from the presynaptic neuron in the tail-withdrawal reflex pathway (1, 2), we wished to show that PKC is actually translocated within sensory cells. After exposing the isolated pleural–pedal ganglia to 5-HT, we dissected out pleural sensory cell clusters and found a 73 ± 14% increase in membrane-bound PKC ($P < 0.02$) and a 27 ± 11% decrease of the kinase in cytosol (Fig. 4), nearly twice the extent of translocation observed with the whole ganglia, which contain neurons other than sensory cells.

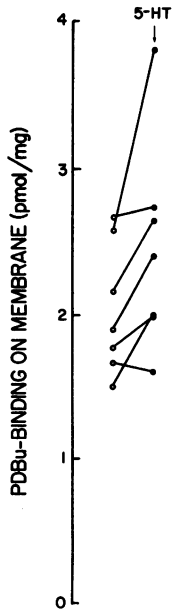

FIG. 3. Translocation of PKC as measured by [^{3}H]phorbol 12,13-dibutyrate (PDBu) binding in isolated pairs of pleural–pedal ganglia. Ganglia were exposed to 5-HT (●) as described; the contralateral (control) ganglia from the same animal were exposed to seawater (connected ○). The average increase was 20 ± 7% ($P < 0.05$).

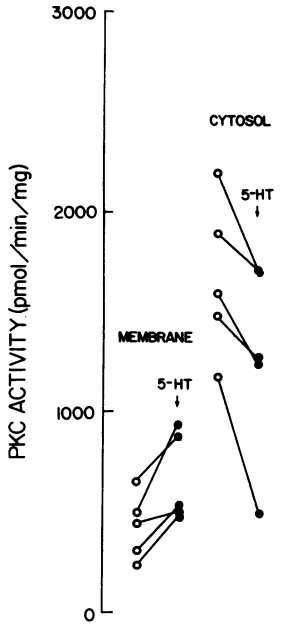

FIG. 4. Translocation of PKC activity in sensory neurons by 5-HT. Kinase activity was increased in the membrane fraction and diminished in the cytosol. ○, Controls; ●, application of 5-HT. Lines drawn between control and experimental points connect the data from the same animals.

DISCUSSION

Demonstrating that PKC is translocated in nervous tissue can be obscured by the high proportion of membrane-bound enzyme found after extraction in buffers containing low concentrations of divalent ions. Mg^{2+} maintains a portion of kinase in the cytosol, however. The concentration of Mg^{2+} used reflects normal intracellular values for marine invertebrates (10 mM). By reducing the proportion of membrane-bound enzyme seen in unstimulated neurons, we were able to detect relatively small shifts in neuronal PKC caused by stimuli that regulate synaptic function.

The extent of PKC translocated in ganglia from sensitized animals is the same as that caused by applying 5-HT to isolated ganglia at a concentration producing maximal presynaptic facilitation. The 40% change in the amount of kinase found to be associated with membrane after sensitization is small relative to the virtually complete translocation produced by phorbol esters in nonneuronal cells—increases of several 100-fold (24). In *Aplysia* neuronal tissue, however, even a maximal dose of phorbol esters results in a relatively small shift of enzyme, only twice that caused by training or by 5-HT. The small shift observed is not likely to result from poor penetration of phorbol esters into the tissue because the same extent of translocation can be demonstrated when the agent is added directly to extracts of the ganglia (25). In contrast, in similar experiments with *Aplysia* buccal muscle, phorbol esters caused a 230% increase of membrane-associated enzyme, a nearly complete translocation of the kinase (K. Weiss, personal communication). Therefore, the amount of PKC capable of being translocated to neuronal membrane is limited, either because there are fewer receptor

sites in neurons than in other tissues or because only a particular neuronal isoform is movable.

A presynaptic role for PKC in enhancing transmitter release has been suggested by the neurophysiological effects of phorbol esters in vertebrates (26–30). But previous experiments on the translocation of the kinase have been limited to studies of long-term potentiation in rat hippocampus (31) and eye-blink conditioning in the rabbit (32) where, enigmatically, the locus of the synaptic change at which PKC is implicated is thought to be primarily postsynaptic (33, 34). The two mechanisms proposed to cause facilitation in *Aplysia* sensory neurons are presynaptic, however: delayed repolarization (3) and enhanced mobilization of transmitter vesicles (5, 6). While abundant evidence indicates that delayed repolarization is produced by cAMP-dependent protein phosphorylation affecting potassium channels (1, 4, 35, 36), its contribution to facilitation diminishes as the synapse becomes depressed (5, 6). Nevertheless, Braha *et al.* [ref. 37 (accompanying paper)] showed that photolysis of caged cAMP can cause facilitation of depressed sensory to motor neuron synapses. On the other hand, phorbol esters enhanced the release of transmitter regardless of prior synaptic activity and without delaying the repolarization of sensory cells.

Our results showing the translocation of PKC in sensory neurons during application of the facilitatory transmitter 5-HT suggest that the presynaptic processes induced by applications of phorbol esters also participate in presynaptic facilitation mediating sensitization and closely related forms of learning such as dishabituation (38). Our experiments do not show how translocation of PKC and phosphorylation of its protein substrates might produce facilitation. Since the application of either phorbol esters or 5-HT increases the spontaneous release of transmitter from sensory cells in culture, however (O. Braha, N. Dale, and E. R. Kandel, personal communication), PKC might make synaptic vesicles more available for exocytosis (29, 39, 40). Our biochemical results are consistent with the idea that the two protein kinases, PKC and PKA (cAMP-dependent protein kinase), act independently in short-term sensitization, dishabituation, or both, through separate molecular mechanisms but towards the same behavioral goal.

We thank Catherine O'Brian and I. Bernard Weinstein for their help with assaying PKC, Heather McMullen for laboratory assistance, and Marc Klein, Vladimir Brezina, Irving Kupfermann, and Eric R. Kandel for reading an earlier version of the manuscript critically.

1. Kandel, E. R. & Schwartz, J. H. (1982) *Science* **218,** 433–443.
2. Walters, E. T., Byrne, J. H., Carew, T. J. & Kandel, E. R. (1983) *J. Neurophysiol.* **50,** 1543–1559.
3. Klein, M. & Kandel, E. R. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 6912–6916.
4. Siegelbaum, S. A., Camardo, J. S. & Kandel, E. R. (1982) *Nature (London)* **299,** 413–417.
5. Hochner, B., Klein, M., Schacher, S. & Kandel, E. R. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 8794–8798.
6. Gingrich, K. J. & Byrne, J. H. (1985) *J. Neurophysiol.* **53,** 652–669.
7. Nishizuka, Y. (1984) *Nature (London)* **308,** 693–698.
8. Scholz, K. P. & Byrne, J. H. (1987) *Science* **235,** 685–687.
9. Greenberg, S. M., Castellucci, V. F., Bayley, H. & Schwartz, J. H. (1987) *Nature (London)* **329,** 62–65.
10. Bernier, L., Castellucci, V. F., Kandel, E. R. & Schwartz, J. H. (1982) *J. Neurosci.* **2,** 1682–1691.
11. O'Brian, C. A., Lawrence, D. S., Kaiser, E. T. & Weinstein, I. B. (1984) *Biochem. Biophys. Res. Commun.* **124,** 296–302.
12. Nishizuka, Y. (1988) *Nature (London)* **334,** 661–665.
13. Uchida, T. & Filburn, C. R. (1984) *J. Biol. Chem.* **259,** 12311–12314.
14. Simpson, I. A. & Sonne, O. (1982) *Anal. Biochem.* **119,** 424–427.
15. Read, S. M. & Northcote, D. H. (1981) *Anal. Biochem.* **116,** 53–64.
16. Niedel, J. E., Kuhn, L. J. & Vandenbark, G. R. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 36–40.
17. Ho, A. K., Thomas, T. P., Chik, C. L., Anderson, W. B. & Klein, D. C. (1988) *J. Biol. Chem.* **263,** 9292–9297.
18. Hodgkin, A. L. (1964) *The Conduction of the Nervous Impulse* (Thomas, Springfield, IL), p. 28.
19. Walters, E. T., Byrne, J. H., Carew, T. J. & Kandel, E. R. (1983) *J. Neurophysiol.* **50,** 1522–1542.
20. Castellucci, V. F. & Kandel, E. R. (1976) *Science* **194,** 1176–1178.
21. Ocorr, K. A. & Byrne, J. H. (1985) *Neurosci. Lett.* **55,** 113–118.
22. Sweatt, J. D. & Kandel, E. R. (1989) *Nature (London)* **339,** 51–54.
23. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257,** 7847–7851.
24. Kraft, A. S. & Anderson, W. B. (1983) *Nature (London)* **301,** 621–623.
25. Sacktor, T. C., Kruger, K. E. & Schwartz, J. H. (1989) *J. Physiol. (Paris)* **83,** 45–52.
26. Tanaka, C., Taniyama, K. & Kusunoki, M. (1984) *FEBS Lett.* **175,** 165–169.
27. Wakade, A. R., Malhotra, R. K. & Wakade, T. D. (1985) *Naunyn Schmiedeberg's Arch. Pharmacol.* **331,** 122–124.
28. Zurgil, N. & Zisapel, N. (1985) *FEBS Lett.* **185,** 257–261.
29. Malenka, R. C., Ayoub, G. S. & Nicoll, R. A. (1987) *Brain Res.* **403,** 198–203.
30. Nichols, R. A., Haycock, J. W., Wang, J. K. T. & Greengard, P. (1987) *J. Neurochem.* **48,** 615–620.
31. Akers, R. F., Lovinger, D. M., Colley, P. A., Linden, D. J. & Routtenberg, A. (1986) *Science* **231,** 587–589.
32. Bank, B., DeWeer, A., Kurizian, A. M., Rasmussen, H. & Alkon, D. L. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 1988–1992.
33. Hu, G.-Y., Hvalby, O., Walaas, S. I., Albert, K. A., Skjeflo, P., Andersen, P. & Greengard, P. (1987) *Nature (London)* **328,** 426–429.
34. Malinow, R., Schulman, H. & Tsien, R. W. (1989) *Science* **245,** 862–866.
35. Walsh, J. P. & Byrne, J. H. (1989) *J. Neurophysiol.* **61,** 32–44.
36. Baxter, D. A. & Byrne, J. H. (1989) *J. Neurophysiol.* **62,** 665–679.
37. Braha, O., Dale, N., Hochner, B., Klein, M., Abrams, T. W. & Kandel, E. R. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 2040–2044.
38. Marcus, E. A., Nolen, T. G., Rankin, C. H. & Carew, T. J. (1988) *Science* **241,** 210–213.
39. Llinas, R., McGuinness, T. L., Leonard, C. S., Sugimori, M. & Greengard, P. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 3035–3039.
40. Goldenring, J. R., Lasher, R. S., Vallano, M. L., Ueda, T., Naito, S., Sternberger, N. H., Sternberger, L. A. & DeLorenzo, R. J. (1986) *J. Biol. Chem.* **261,** 8495–8504.