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Muscarinic agonists and phorbol esters increase tyrosine phosphorylation of a 40-kilodalton protein in hippocampal slices

(protein kinase C/phosphotyrosine proteins/inositol phospholipid system/muscarinic receptors)

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ABSTRACT **We have used the hippocampal slice preparation to investigate the regulation of protein tyrosine phosphorylation in brain. After pharmacological treatment of intact slices, proteins were separated by electrophoresis, and levels of protein tyrosine phosphorylation were assessed by immunoblotting with specific anti-phosphotyrosine antibodies. Phorbol esters, activators of the serine- and threonine-phosphorylating enzyme protein kinase C, selectively increase tyrosine phosphorylation of a soluble protein with an apparent molecular mass of approximately 40 kilodaltons. Muscarinic agonists such as carbachol and oxotremorine M that strongly activate the inositol phospholipid system also increase tyrosine phosphorylation of this protein. Neurotransmitter activation of the inositol phospholipid system and protein kinase C appears to trigger a cascade leading to increased tyrosine phosphorylation.**

In recent years, a group of protein kinases has been identified that selectively phosphorylates proteins on tyrosine residues (1). These phosphotyrosine residues are much less abundant than phosphoserine or phosphothreonine residues, accounting for <1% of phosphoamino acid residues in many cell types. Despite the low levels of phosphotyrosine-containing proteins, tyrosine-specific protein kinases play a major role in regulating cell processes, since several oncogene products and growth factor receptors possess tyrosine-specific protein kinase activity critical for influencing cell growth and proliferation (1, 2).

Tyrosine kinase activity is particularly abundant in adult brain (3, 4). Paradoxically, it is associated with neurons, a nonproliferating cell type (5–8), indicating involvement in aspects of neuronal function other than cell proliferation. Several synaptic vesicle proteins (7) and the nicotinic acetylcholine receptor (9) have been identified as substrates for tyrosine phosphorylation. These findings point to a role of tyrosine-specific kinases in synaptic transmission, yet little is known about the regulation of tyrosine phosphorylation in neuronal systems. In the present study, we have used the hippocampal slice preparation to examine this question. We have found that tyrosine phosphorylation of a 40-kilodalton (kDa) protein is increased by both phorbol esters and muscarinic receptor activation, indicating that tyrosine phosphorylation may mediate some responses to neurotransmitters and protein kinase C activation.

MATERIALS AND METHODS

Hippocampal Slice Preparation. Rat hippocampal slices were prepared in a standard fashion (10) from adult male Sprague–Dawley rats (150–250 g). Hippocampi were dissected, and 400-μm-thick transverse slices were cut with a manual chopper. Slices were transferred to a chamber containing a humidified atmosphere of 5% CO_2/95% O_2. Slices rested on filter paper covering a small dish of physiological saline (130 mM NaCl/5.0 mM KCl/24 mM $NaHCO_3$/2.5 mM $CaCl_2$/1.5 mM $MgSO_4$/1.2 mM NaH_2PO_4/10 mM glucose). Slices were allowed to recover for at least 1 hr prior to transferring them to dishes with drug-containing salines. Oxotremorine M and oxotremorine 2 were gifts from S. K. Fisher (Ann Arbor, MI). Other drugs were obtained from standard commercial sources.

Antibody Preparation and Immunoblot Analysis. Antisera against phosphotyrosine were prepared as described by Ohtsuka *et al.* (11). In brief, phosphotyrosine coupled to either keyhole limpet protein or bovine serum albumin was injected intradermally. The resulting antisera were affinity-purified on a Sepharose 4B column coupled to phosphotyrosine. Monoclonal antibodies to phosphotyrosine were obtained commercially (ICN).

After drug treatments, individual slices were added to 150 μl of stop buffer [2% sodium dodecyl sulfate/125 mM Tris·HCl, pH 6.8/10% (wt/vol) glycerol/5% 2-mercaptoethanol/1 mM sodium orthovanadate], sonicated briefly, and placed in boiling water for 2 min. Proteins were separated by electrophoresis on sodium dodecyl sulfate/7.5% polyacrylamide gels (SDS/PAGE) by the method of Laemmli (12). The proteins were then transferred to nitrocellulose sheets at 200 mA overnight by using the buffer system of Towbin *et al.* (13). Immunoblotting with the anti-phosphotyrosine antibody was performed with ^{125}I-labeled protein A as described by Jahn *et al.* (14), except that a 1:1000 dilution of the antibody was used. Autoradiograms were generated by exposing the blot to Kodak XAR film.

In some experiments, soluble and particulate fractions were separated prior to SDS/PAGE. After drug treatments, two slices were added to 200 μl of 50 mM Tris·HCl, pH 7.4/100 mM NaF/50 mM NaCl/10 mM EGTA/5 mM EDTA/10 mM sodium pyrophosphate/20 mM sodium phosphate/1 mM sodium orthovanadate/20 μg of leupeptin per ml/20 μg of antipain per ml/20 units of Trasylol per ml, sonicated, and then spun at 75,000 rpm in a TL-100 Beckmann centrifuge for 15 min. The pellets were washed and then resuspended in 200 μl of the same buffer. One hundred microliters of triple-strength stop buffer was added to both soluble and particulate fractions prior to loading half of each sample per lane.

RESULTS

In untreated hippocampal slices, several phosphotyrosine-containing proteins were detectable when homogenates of the slices were analyzed by immunoblotting techniques with antibodies specific for phosphotyrosine. Several prominently labeled proteins were apparent with molecular masses of approximately 175, 125, 115, and 95 kDa (Fig. 1). Incubation of hippocampal slices with 5 μM phorbol 12,13-diacetate, a phorbol ester analogue that activates protein kinase C (15),

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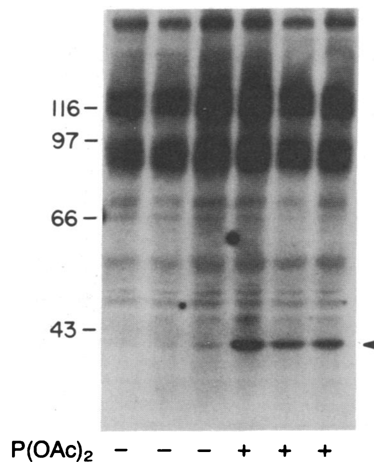

FIG. 1. Phorbol ester-induced increase in tyrosine phosphorylation of a 40-kDa protein. Hippocampal slices were incubated in control saline or 5 μM phorbol 12,13-diacetate [P(OAc)$_2$] for 1 hr. Proteins were separated by SDS/PAGE, and anti-phosphotyrosine antibodies were used to detect phosphotyrosine-containing proteins. Slices exposed to phorbol 12,13-diacetate display increased tyrosine phosphorylation of a protein migrating with an approximate molecular mass of 40-kDa (arrowhead). Individual hippocampal slices were run in each lane.

selectively increased tyrosine phosphorylation of a protein migrating with an apparent molecular mass of approximately 40 kDa (Fig. 1). The labeling of proteins with the anti-phosphotyrosine antibody was completely blocked by preincubation of the antibody with 200 μM phosphotyrosine but not 200 μM phosphoserine or phosphothreonine. In addition, a similar pattern of labeling was detected by affinity-purified antibodies generated to phosphotyrosine coupled to either keyhole limpet protein or bovine serum albumin and by an anti-phosphotyrosine monoclonal antibody (16).

To analyze whether the 40-kDa protein is soluble or membrane-associated, the soluble and particulate fractions were separated prior to SDS/PAGE. The 40-kDa protein was recovered almost entirely in the soluble fraction (Fig. 2). Of the other prominently labeled proteins, the 95-kDa protein was detected exclusively in the particulate fraction, and the 175-, 125-, and 115-kDa proteins appeared in both fractions.

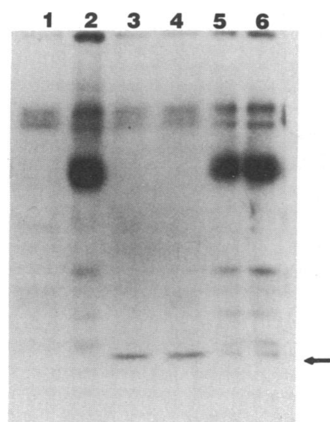

FIG. 2. Separation of soluble and particulate phosphotyrosine proteins. Soluble and particulate fractions were separated prior to SDS/PAGE. The 40-kDa protein displaying increased tyrosine phosphorylation after exposure to phorbol 12,13-diacetate is found largely in the soluble fraction (arrow). Lanes: 1 and 2, soluble and particulate fractions, respectively, from control slices; 3–6, 5 μM phorbol 12,13-diacetate-treated (1 hr) slices (soluble fractions were run in lanes 3 and 4, and particulate fractions, in lanes 5 and 6).

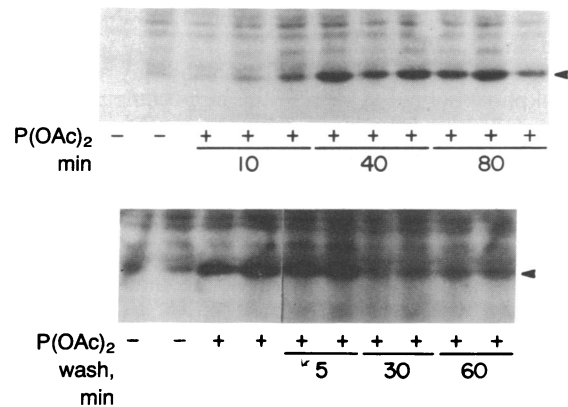

FIG. 3. (*Upper*) Time course of phorbol ester-induced increase in tyrosine phosphorylation. After incubation of hippocampal slices with phorbol 12,13-diacetate [P(OAc)$_2$] for the times indicated, slices were placed in stop buffer and subjected to immunoblot analysis with anti-phosphotyrosine antibodies. As shown in the top panel, little increase in labeling in the 40-kDa band is apparent at 10 min. By 40 min, a substantial increase appears that is sustained at 80 min. (*Lower*) The enhanced tyrosine phosphorylation is readily reversible. Slices were exposed to 5 μM P(OAc)$_2$ for 1 hr and then transferred to control saline for the times indicated. A decrease to baseline levels is seen with washing for either 30 or 60 min. This decrease is not simply due to transferring the slices *per se* because after 5 min of washing, which does not allow sufficient time for removal of the drug, a decrease in staining was not observed.

The increase in phosphotyrosine phosphorylation was barely detectable at 10 min but was clearly apparent after 40 min and remained elevated for several hours (Fig. 3). This response to phorbol esters is reversible, as transfer of slices from buffer containing phorbol 12,13-diacetate to control saline returned the phosphotyrosine phosphorylation of the 40-kDa protein to control levels (Fig. 3). Accordingly, persistent activation of protein kinase C was required to maintain the increased phosphotyrosine content of this protein.

The concentration–response relationship for phorbol 12,13-diacetate was examined after incubation of slices for 1 hr. A small increase of phosphotyrosine phosphorylation occurred at 0.5 μM, with maximal increases noted at 2 and 5 μM (Fig. 4). Because phorbol ester analogues vary widely in their affinities for protein kinase C (15), we examined the activity of the phorbol ester analogues phorbol 12,13-dibutyrate, phorbol 12,13,20-triacetate, and phorbol in mimicking this response. The rank order of potencies of these drugs paralleled their affinities for protein kinase C: 100 nM phorbol 12,13-dibutyrate was more active than a similar concentration of phorbol 12,13-diacetate, phorbol 12,13,20-

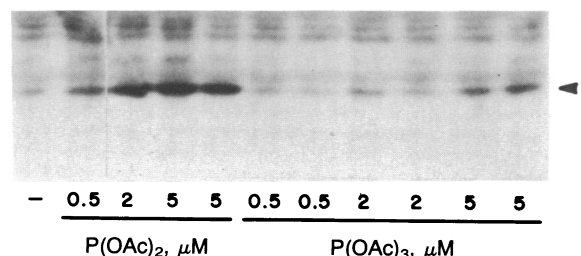

FIG. 4. Concentration–response relationship for phorbol esters. The effects of several concentrations of phorbol 12,13-diacetate [P(OAc)$_2$] and phorbol 12,13,20-triacetate [P(OAc)$_3$] were examined after 1 hr of incubation. P(OAc)$_2$ at 0.5 μM produces a small increase in labeling of the 40-kDa protein, whereas 2 μM elicits a larger increase, with little further effect at 5 μM. P(OAc)$_3$ also shows a concentration-dependent increase in staining but is less potent than P(OAc)$_2$.

triacetate was less potent than phorbol 12,13-diacetate (Fig. 4), and phorbol was completely inactive at 10 μM.

As muscarinic receptor stimulation strongly activates the inositol phospholipid system in the hippocampal slice preparation (17), we wondered whether muscarinic agonists would also elicit a similar increase in anti-phosphotyrosine labeling of the 40-kDa protein. In the hippocampus, as in N1E-115 neuroblastoma cells, muscarinic receptors with low affinity for agonists mediate increases in inositol phospholipid turnover (17–20). In both these preparations, concentrations of 1 mM carbachol are needed to elicit near-maximal stimulation, with little effect observed below 50–100 μM. Carbachol exhibited a similar profile in eliciting increased tyrosine phosphorylation of the 40-kDa protein. Incubation of slices with 1 mM carbachol for 15–30 min produced a marked increase in staining (Fig. 5), with only small increases apparent with 100 μM. The effects of 1 mM carbachol were blocked by atropine (1–10 μM, Fig. 6).

Muscarinic stimulation of the inositol phospholipid system in the hippocampus displays a characteristic pharmacology (10, 17, 18, 21). Several muscarinic agonists such as carbachol, oxotremorine M, and oxotremorine 2 possess high efficacy, while others such as oxotremorine, arecoline, and pilocarpine behave as weak partial agonists. These agents displayed a similar profile in their ability to increase the phosphotyrosine content of the 40-kDa protein. When tested at a concentration of 1 mM, oxotremorine M (Fig. 6) and oxotremorine 2 mimicked carbachol's action, while oxotremorine, arecoline, and pilocarpine did not. In addition, oxotremorine blocked increases produced by oxotremorine M (Fig. 6).

To assess the role of protein kinase C in mediating carbachol's effect on tyrosine phosphorylation, we examined the effects of an inhibitor of protein kinase C, H-7 (22). In hippocampal slices, relatively high concentrations (300 μM) and long incubations (>1 hr) are required to allow for penetration of this lipophilic compound (23). Under these conditions, we found that H-7 blocked both phorbol 12,13-diacetate (5 μM for 60 min)- and carbachol (1 mM for 15 min)-induced increases in tyrosine phosphorylation of the 40-kDa protein, as would be expected if protein kinase C mediated these effects. However, H-7 is not specific for protein kinase C, leaving open the possibility that it directly inhibits the tyrosine-specific protein kinase involved or that carbachol may act via another H-7-sensitive protein kinase.

DISCUSSION

Our results demonstrate regulation of tyrosine phosphorylation by phorbol esters, activators of the serine- and threonine-phosphorylating enzyme protein kinase C, and by muscarinic receptor activation. In particular, we have identified a soluble 40-kDa protein that is selectively tyrosine-phosphorylated in response to these agents. Abundant evidence points to protein kinase C as the primary target of phorbol esters (15); however, other sites of actions have been postulated including other kinases (40, 41). Since muscarinic agonists that stimulate the inositol phospholipid–protein kinase C cascade mimic this response to phorbol esters, it appears likely that protein kinase C mediates this effect. As protein kinase C is not a member of the tyrosine kinase family (1), we infer that activated protein kinase C triggers a cascade leading to increased tyrosine phosphorylation of this protein. Precedent for an indirect increase in protein tyrosine phosphorylation by protein kinase C has been observed in fibroblasts in culture. In these cells, phorbol esters increase tyrosine phosphorylation of a soluble 42-kDa protein that is also regulated by several growth factors (1, 24–26). It appears likely that the 40-kDa protein we have identified in brain is related to the fibroblast protein. By contrast, the 40-kDa protein is distinct from pp38 or synaptophysin, a known tyrosine kinase substrate in brain, since that protein is an integral membrane protein (7). Another candidate is calpactin I, a 38-kDa tyrosine kinase substrate, present in low levels in brain (42, 43). However, parallel Western blots stained with antisera for calpactin show that they migrate differently (unpublished observation).

Muscarinic receptors are linked to multiple second-messenger pathways (27, 28). The distinctive agonist profile we have observed for increases in tyrosine phosphorylation of the 40-kDa protein closely resembles that reported for stimulation of inositol phospholipid turnover in the hippocampus (20). Since phorbol esters mimic this cholinergic response, it appears likely that muscarinic effects on tyrosine phosphorylation are mediated via the inositol phospholipid system and protein kinase C and that the activation of a protein tyrosine kinase is a general consequence of protein kinase C activation.

As muscarinic agonists and protein kinase C affect several membrane ionic conductances in hippocampal pyramidal neurons (29, 30), the 40-kDa protein we have identified may be involved in mediating some of these actions. In pyramidal neurons, phorbol esters and muscarinic agonists that stimu-

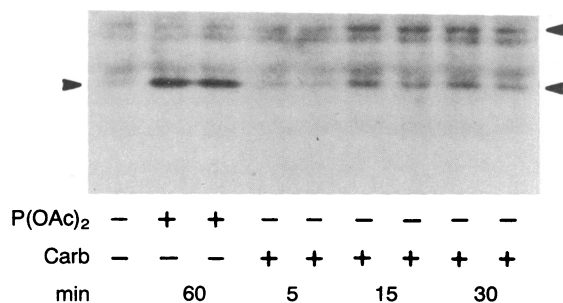

FIG. 5. Effects of carbachol (Carb) on tyrosine phosphorylation. Slices were incubated with 1 mM carbachol for 5, 15, or 30 min and then were processed for immunoblotting with anti-phosphotyrosine antibodies. Increases are apparent at 15 and 30 min, but not at 5 min, in the 40-kDa band (lower arrowhead). The response to phorbol 12,13-diacetate [P(OAc)$_2$] (5 μM for 60 min) is shown for comparison. Smaller and more variable increases in one of the more slowly migrating bands were also observed (upper arrowhead).

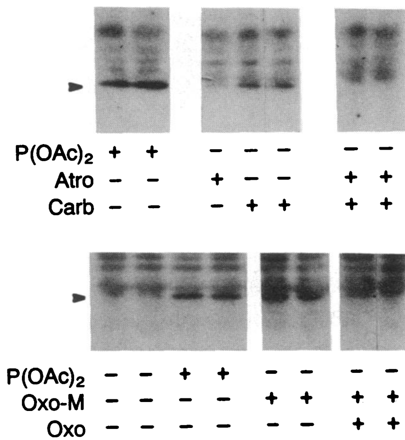

FIG. 6. Pharmacology of muscarinic effects on tyrosine phosphorylation. (*Upper*) Pretreatment of slices with 2 μM atropine for 1 hr blocks the ability of 1 mM carbachol (Carb) to increase tyrosine phosphorylation of the 40-kDa band. Incubation with 10 μM atropine (Atro) alone does not affect the staining pattern. The effect of phorbol 12,13-diacetate [P(OAc)$_2$] (5 μM for 60 min) is shown in the left-hand lanes for comparison. (*Lower*) Oxotremorine M (Oxo-M; 1 mM for 15 min) mimics carbachol's effect on the 40-kDa band. Oxotremorine (Oxo; 1 mM), a muscarinic agonist with low efficacy at stimulating inositol phospholipid turnover, blocks 1 mM oxotremorine M.

late inositol phospholipid turnover share a common action. They block the inhibitory effects of several receptors coupled to potassium channels via pertussis-toxin-sensitive GTP-binding proteins (10, 31). For example, potassium-channel activation elicited by adenosine or serotonin is blocked by these agents. The concentrations of phorbol esters required to block adenosine (32) closely match those effective in eliciting tyrosine phosphorylation. Accordingly, the 40-kDa protein may be involved in modulation of neuronal signaling by protein kinase C.

Recent studies have linked protein kinase C to processes underlying synaptic plasticity. In hippocampal neurons, protein kinase C activation mimics several aspects of long-term potentiation (33, 34), and inhibitors of protein kinase C such as H-7 (22) block this form of synaptic potentiation (23, 35, 36). Since phorbol esters increase tyrosine phosphorylation of the 40-kDa protein and this effect is blocked by H-7, it is possible that this substrate protein may be involved in regulating synaptic plasticity. In considering possible roles of tyrosine phosphorylation in synaptic plasticity, it may be relevant that tyrosine phosphorylation plays a key role in mediating rapid expression of genes after cell-surface receptor stimulation (2). This type of rapid genomic response has been demonstrated in hippocampal neurons (37, 38) and is thought to be critical for long-term changes in synaptic responses (39). Accordingly, tyrosine phosphorylation secondary to protein kinase C activation demonstrated in this study could be an important step in a cascade regulating neuronal genomic responses.

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1. Hunter, T. & Cooper, J. A. (1985) *Annu. Rev. Biochem.* **54**, 897–930.
2. Yarden, Y. & Ullrich, A. (1988) *Annu. Rev. Biochem.* **57**, 443–478.
3. Cotton, P. C. & Brugge, J. S. (1983) *Mol. Cell. Biol.* **3**, 1157–1162.
4. Sudol, M. & Hanafusa, H. (1986) *Mol. Cell. Biol.* **6**, 2839–2846.
5. Hirano, A. A., Greengard, P. & Huganir, R. L. (1988) *J. Neurochem.* **50**, 1447–1455.
6. Walaas, S. I., Lustig, A., Greengard, P. & Brugge, J. S. (1988) *Mol. Brain Res.* **3**, 215–222.
7. Pang, D. T., Wang, J. K. T., Valtora, F., Benfenati, F. & Greengard, P. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 762–766.
8. Ellis, P. D., Bissoon, N. & Gurd, J. W. (1988) *J. Neurochem.* **51**, 611–620.
9. Huganir, R. L., Miles, K. & Greengard, P. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6968–6972.
10. Worley, P. F., Baraban, J. M., McCarren, M., Snyder, S. H. & Alger, B. E. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 3467–3471.
11. Ohtsuka, M., Ihara, S., Ogawa, R., Watanabe, T. & Watanabe, Y. (1984) *Int. J. Cancer* **34**, 855–861.
12. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
13. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
14. Jahn, R., Schiebler, W. & Greengard, P. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1684–1687.
15. Blumberg, P. M., Jaken, S., Konig, B., Sharkey, N. A., Leach, K. L., Jeng, A. Y. & Yeh, E. (1984) *Biochem. Pharmacol.* **33**, 933–940.
16. Glenney, J. R., Jr., Zokas, L. & Kamps, M. P. (1988) *J. Immunol. Methods* **109**, 277–285.
17. Fisher, S. K., Klinger, P. D. & Agranoff, B. W. (1983) *J. Biol. Chem.* **258**, 7358–7363.
18. Fisher, S. K. & Snider, R. M. (1987) *Mol. Pharmacol.* **32**, 81–90.
19. McKinney, M., Stenstrom, S. & Richelson, E. (1985) *Mol. Pharmacol.* **27**, 223–235.
20. Fisher, S. K. & Bartus, R. T. (1985) *J. Neurochem.* **45**, 1085–1095.
21. Fisher, S. K., Figueiredo, J. C. & Bartus, R. T. (1984) *J. Neurochem.* **43**, 1171–1179.
22. Kawamoto, S. & Hidaka, H. (1984) *Biochem. Biophys. Res. Commun.* **125**, 258–264.
23. Malinow, R., Madison, D. V. & Tsien, R. W. (1988) *Nature (London)* **335**, 820–824.
24. Gilmore, T. & Martin, G. S. (1983) *Nature (London)* **306**, 487–490.
25. Bishop, R., Martines, R., Nakamora, K. D. & Weber, M. J. (1983) *Biochem. Biophys. Res. Commun.* **115**, 536–543.
26. Cooper, J. A. & Hunter, T. (1985) *Mol. Cell. Biol.* **5**, 3304–3309.
27. McKinney, M. & Richelson, E. (1984) *Annu. Rev. Pharmacol. Toxicol.* **24**, 121–146.
28. Nathanson, N. M. (1987) *Annu. Rev. Neurosci.* **10**, 195–236.
29. Baraban, J. M., Snyder, S. H. & Alger, B. E. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 2538–2542.
30. Malenka, R. C., Madison, D. V., Andrade, R. & Nicoll, R. A. (1986) *J. Neurosci.* **6**, 475–480.
31. Andrade, R., Malenka, R. C. & Nicoll, R. A. (1986) *Science* **234**, 1261–1265.
32. Stratton, K. R., Baraban, J. M. & Worley, P. F. (1988) *Synapse* **2**, 614–618.
33. Hu, G.-Y., Hvalby, O., Walaas, S. I., Albert, K. A., Skjeflo, P., Anderse, P. & Greengard, P. (1987) *Nature (London)* **328**, 426–429.
34. Malenka, R. C., Madison, D. V. & Nicoll, R. A. (1986) *Nature (London)* **321**, 175–177.
35. Kauer, J. A., Malenka, R. C. & Nicoll, R. A. (1988) *Nature (London)* **334**, 250–252.
36. Lovinger, D. M., Wong, K. W., Murakami, D. & Routtenberg, A. (1987) *Brain Res.* **436**, 177–183.
37. Morgan, J. I., Cohen, D. R., Hempstead, J. L. & Curran, T. (1987) *Science* **237**, 192–197.
38. Saffen, D. W., Cole, A. J., Worley, P. F., Christy, B. A., Ryder, K. & Baraban, J. M. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7795–7799.
39. Goelet, P., Castellucci, V. F., Schacher, S. & Kandel, E. R. (1986) *Nature (London)* **322**, 419–422.
40. Greenberger, G., Zick, Y., Taylor, S. I. & Gorden, P. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 2762–2766.
41. Moon, S. O., Palfrey, H. C. & King, A. C. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 2298–2302.
42. Crompton, M. R., Moss, S. E. & Crumpton, M. J. (1988) *Cell* **55**, 1–3.
43. Pepinsky, R. B., Tizard, R., Mattaliano, R. J., Sinclair, L. K., Miller, G. T., Browning, J. L., Chow, E. P., Burne, C., Huang, K.-S., Pratt, D., Wachter, L., Hession, C., Frey, A. Z. & Wallner, B. P. (1988) *J. Biol. Chem.* **263**, 10799–10811.