Protein kinase C and F1/GAP-43 gene expression in hippocampus inversely related to synaptic enhancement lasting 3 days

(long-term potentiation/CA3/*in situ* hybridization)

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ABSTRACT The mRNA levels of protein F1 (also known as GAP-43), and protein kinase C (PKC) subtypes were measured 3 days after the induction of long-term enhancement (also known as long-term potentiation) in the hippocampus of chronically prepared conscious rats by quantitative *in situ* hybridization. Altered mRNA levels correlated significantly with alternations in synaptic efficacy; such correlations have not been reported previously. Rats with greater synaptic enhancement had lower gene expression in the CA3 subfield of F1/GAP-43 and both β -PKC and γ -PKC, but not α -PKC. For microtubule-associated protein 2 (MAP-2), neurogranin, and the glutamate receptor subtype B-flip, no correlation was observed in any cell field between synaptic enhancement and hybridization to the mRNA. To our surprise, alterations in mRNA levels of F1/GAP-43 and γ -PKC were highly correlated ($r = +0.928$, $P < 0.001$), suggesting coordinate regulation. Since F1/GAP-43 is associated with neurite growth, its lowered expression at 3 days would reduce potential growth, leading to synaptic stabilization. We propose that long-term synaptic change is mediated by gene expression of the very same proteins initially modified posttranslationally.

Protein kinase C (PKC) activity is increased after the induction of long-term enhancement (LTE; also known as long-term potentiation, LTP) of synaptic transmission (1, 2). Because PKC activation is critical for the persistence of LTE lasting hours, and increased phosphorylation of the PKC substrate F1/GAP-43 (43-kDa growth-associated protein, also known as B-50, neuromodulin, or pp46) is directly related to LTE (3, 4), it is possible that longer-term persistence, measured in days, is mediated by altered gene expression of these very same proteins. To investigate this possibility, LTE induction was performed in chronically implanted rats given perforant path stimulation and monitored for 3 days, after which quantitative *in situ* hybridization in hippocampus was performed.

MATERIALS AND METHODS

Experimental Treatments. Adult rats were tested in four separate experiments. In the first two experiments ($n = 6$ rats in each group) rats received bilateral stimulation. High-frequency stimulation (HFS) was given to one hemisphere, producing LTE, and the other side was given low-frequency stimulation (LFS), which does not significantly change baseline responses. The results from the second experimental group replicated the first and are combined here for brevity. In the third experiment electrodes were again implanted bilaterally, but only unilateral stimulation, either HFS ($n = 5$) or LFS ($n = 4$), was given. In the fourth experiment animals

received bilateral stimulation but were analyzed at 2, 12, or 24 hr ($n = 4$ at each time) after the final HFS.

Chronic electrophysiological recordings were performed as described (3), with minor modifications. Male F-344 rats were anesthetized with Nembutal (33 mg/kg) prior to surgery, and Children's Tylenol was administered as an analgesic for 3 days after surgery in the drinking water. Bilateral stimulating electrodes were placed in the angular bundle and bilateral recording electrodes in the dentate gyrus hilar/CA3c region. The side receiving specific stimulation was counter-balanced among animals. Stimulus intensity was adjusted to give a one-third maximal population spike response. A total of 100 pulses were delivered at 400 Hz (10- or 25-msec bursts) for the HFS treatment. The same total number of pulses were given for the LFS treatment at a rate of 2 Hz. To achieve maximal persistence of LTE, HFS was given three times each day for two consecutive days. Because there are direct perforant path inputs to both dentate granule cells and pyramidal cells of CA3 and CA1 (5), and high-frequency stimulation of the perforant path induces LTE in both pathways (6), LTE recorded from the dentate gyrus/CA3c region was used as an index of synaptic enhancement for both granule and pyramidal cell fields. At the end of the electrophysiological experiments, brains were coded and the blind was not broken until after quantitation of the results.

In Situ Hybridization. *In situ* hybridization with RNA probes was performed as described (7), with an acetic anhydride pretreatment and stringent washing in $0.1 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7) at 60°C. Sections hybridized with oligonucleotides were pretreated as above and defatted for 5 min in chloroform, and hybridization and washes were performed as described (8), with the following modifications. Dextran sulfate (10%, wt/vol) was added to the hybridization buffer. Sections were washed in $1 \times$ SSC/0.05% sodium pyrophosphate/20 mM 2-mercaptoethanol, with the stringent wash at 55°C for 60–90 min. Control sections were hybridized to the sense strand when RNA probes were used. For oligonucleotide probes, control sections were either RNase-treated or hybridized with a 30-fold excess of unlabeled oligonucleotides. The hybridization observed in the hippocampus was consistent with prior reports for all probes used (Fig. 1).

Oligonucleotide sequences unique to specific subtype mRNAs were synthesized by the Biotechnology Facility at Northwestern University. The B-flip probe corresponded to residues 775–787 of the “flip” form of the glutamate receptor GluR-B (12). The γ -PKC and β -PKC probes were complementary to bases 2286–2333 of the PKC-I cDNA and bases 2167–2214 of the PKC-II cDNA, respectively (13). The α -PKC probe was complementary to bases 679–726 of the cDNA (14).

Fig. 1. Distribution of mRNAs assayed by *in situ* hybridization. The cellular distribution of *in situ* hybridization in control hippocampus was consistent with prior reports for all probes used. (A) γ -PKC hybridization was equivalently high in all pyramidal cell fields and lower in granule cells (18). The section shown is from a rat which received unilateral HFS. Note the decreased labeling in CA3 on the right side compared to the left side, which received no stimulation. The approximate boundaries of CA3 are indicated with arrowheads. (B) F1/GAP-43 hybridization was highest in CA3 and CA4 subfields, lower in CA1 and CA2, and absent from the dentate granule cells (7). This animal received HFS on the right side and LFS on the left. Note the decreased labeling in CA3 on the HFS side compared to the LFS side. (C) α -PKC hybridization was generally uniform throughout the hippocampus, but somewhat higher in CA2 and CA3a than other cell fields. (D) β -PKC hybridization was highest in CA1, absent from CA2, and low in granule cells (18). The approximate boundaries of the CA2 cell field are indicated by triangles. (E) Microtubule-associated protein 2 (MAP-2) hybridization levels were similar in the granule cells and pyramidal cell fields, and the hybridization extended into the dendrites, being maximal in proximal dendritic zones of CA3 (10). (F) Neurogranin hybridization was similar in all hippocampal neurons (11). (G) B-flip glutamate receptor hybridization was slightly stronger over pyramidal cell fields than over granule cells (12). (H) In a control section, hybridization with a 30-fold excess of unlabeled probe blocked hybridization to the 35 S-labeled γ -PKC oligonucleotide probe. Specificity of hybridization for all probes was demonstrated by absence of hybridization on control sections (see *Materials and Methods*). (Bar = 1 mm.)

The following RNA probes were used: MAP-2, corresponding to bases 2320–2850 of the cDNA clone 19a (15); neurogranin, containing the coding region for the first 30 amino acids and the 5' untranslated region of the RC3 cDNA (11); and F1/GAP-43, corresponding to bases 779–1295 of the rat cDNA (16). The specificity of the probes was verified by hybridization to the proper-sized transcripts on Northern blots.

Quantitative Analysis. Quantitation was performed by image analysis of areal grain densities as described (17). Eight to 10 sections over the dorsal hippocampus were analyzed per animal. Computer-derived areal densities were linearly related to a sampling of manually derived grain counts (coefficient of correlation, $r = +0.996$). Unless otherwise noted, quantitation results are reported as percent of the contralateral, control side. Comparison between hemispheres was done to reduce procedure-dependent variation and therefore increase the sensitivity of the analyses.

RESULTS

In both the first and second experiments, the greater the magnitude of synaptic enhancement, the lower was the level of hybridization. This was observed in the CA3 subfield for F1/GAP-43, β -PKC, and γ -PKC. With a >20% increase in excitatory postsynaptic potential (EPSP) magnitude selected as a criterion for LTE persistence, animals which exhibited persistent LTE ($n = 6$) had significantly lower F1/GAP-43 hybridization ($P < 0.05$) and γ -PKC hybridization ($P < 0.01$) than animals which received HFS but did not express persistent LTE ($n = 6$; Fig. 2A). Although β -PKC hybridization was somewhat lower in animals exhibiting LTE, the difference was not significant ($P > 0.50$).

Persistence of LTE was inversely related to F1/GAP-43 ($r = -0.801$, $P < 0.01$; Fig. 2B) and γ -PKC ($r = -0.781$, $P < 0.01$; Fig. 2C) hybridization and nonsignificantly to β -PKC ($r = -0.692$, $P < 0.058$) in the CA3 subfield. α -PKC hybridization in CA3 exhibited no significant relation to synaptic enhancement ($r = +0.154$, $P > 0.75$).

Comparisons of areal grain density and EPSP changes on the HFS side only (e.g., for the first experimental group) showed the same such inverse correlations between synaptic enhancement and both F1/GAP-43 ($r = -0.789$) and γ -PKC ($r = -0.545$) hybridization. The correlations were thus most likely a product of alterations on the HFS side rather than on the LFS side. To test this explicitly, we performed a third experiment in which only unilateral stimulation (either LFS or HFS) was given. We again found a negative correlation between F1/GAP-43 hybridization and magnitude of EPSP increase in the CA3 subfield ($r = -0.758$, $P < 0.05$; Fig. 3B). HFS caused a decrease in hybridization in comparison to the unstimulated side (87% of control side; $P < 0.05$, paired t test; Fig. 3A), whereas LFS did not (+114%; $P = 0.23$). When raw hybridization values were compared directly between the stimulated sides of the two treatment groups, the HFS animals exhibited 25% lower F1/GAP-43 hybridization in CA3 than LFS animals. There was only a 1% difference between groups in F1/GAP-43 hybridization on the contralateral, unstimulated side. The one HFS animal that showed no decrease in hybridization also had the least EPSP increase, which is consistent with the first two experiments (compare Fig. 2A with Fig. 3B). Similar results were also obtained for γ -PKC after unilateral stimulation (Fig. 1A).

A surprisingly high correlation existed between stimulation-induced alterations in mRNA expression of F1/GAP-43 and γ -PKC in CA3 ($r = +0.928$, $P < 0.001$; Fig. 2D). Relative changes in F1/GAP-43 and γ -PKC mRNA expression were also correlated in CA1 ($r = +0.707$, $P < 0.05$). In contrast, the basal distribution and relative expression levels of these two mRNAs are dissimilar (Fig. 1). A positive correlation was also observed between F1/GAP-43 and β -PKC in CA3 ($r = +0.765$, $P < 0.05$) but not in CA1 ($r = +0.530$, $P = 0.18$). Since F1/GAP-43 is preferentially phosphorylated by β -PKC (20), the higher correlation between F1/GAP-43 and γ -PKC was unexpected.

The significant decrease after LTE in mRNA for F1/GAP-43 and γ -PKC was selective for hippocampal subfield CA3, consistent with its postulated central role in memory

FIG. 2. Gene expression in subfield CA3 of the hippocampus related to 3-day synaptic enhancement. (A) Animals which expressed persistent LTE, as defined as a $>20\%$ increase in EPSP magnitude, expressed lower F1/GAP-43 hybridization in CA3 than those animals which received HFS but did not express persistent LTE. (B) F1/GAP-43 hybridization is inversely related to synaptic enhancement. (C) γ -PKC hybridization is also inversely related to synaptic enhancement. (D) LTE-dependent alterations in hybridization levels for F1/GAP-43 and γ -PKC over CA3 are highly correlated. Hybridization levels are reported as the percent difference in areal grain density between the side receiving HFS and the side receiving LFS; the EPSP change is the average fractional change from baseline recordings. Comparisons were made between hemispheres to minimize procedure-dependent variation (due to tissue processing, hybridization, and autoradiography) and to enhance the sensitivity of the assay. The fractional change in EPSP magnitude on the control side (LFS) was subtracted from the fractional change on the HFS side in order to minimize variation due to environmental effects on the EPSP, such as short-term exploratory or thermal modulation effects on the EPSP (19, 36).

function (21, 22). No significant correlation existed between synaptic enhancement and γ -PKC hybridization over granule cells ($r = -0.486$, $P > 0.10$) or the CA1 subfield ($r = -0.300$; $P > 0.30$) or between synaptic enhancement and β -PKC hybridization over granule cells ($r = -0.413$, $P > 0.30$) or CA1 ($r = -0.530$, $P < 0.20$). F1/GAP-43 hybridization over CA1 also did not correlate with synaptic enhancement ($r = +0.088$; $P > 0.75$). While hippocampal granule cells do not normally express F1/GAP-43 (7), expression can be robustly induced by seizure activity (17); perhaps less intense synaptic activation would also cause induction. However, HFS did not elevate F1/GAP-43 hybridization in granule cells above background levels (Fig. 1B).

No significant relation was observed between LTE and neurogranin, MAP-2, or B-flip hybridization over CA3, CA1, or granule cells. Analysis of MAP-2 hybridization restricted to the dendritic zones of CA3, CA1, or granule cells also did

not correlate with LTE. There was a 13% greater B-flip hybridization on the HFS side in granule cells ($P < 0.05$, paired t test), which did not, however, correlate with LTE ($r = -0.158$; $P > 0.60$).

We analyzed animals 2, 12, and 24 hr after the final HFS treatment. F1/GAP-43 mRNA expression was not increased in CA3, and no induction was observed in granule cells at any of these earlier time points.

DISCUSSION

In three separate experiments we have observed an inverse correlation between F1/GAP-43 gene expression and synaptic enhancement 3 days after HFS. Since increases in F1/GAP-43 mRNA and protein levels are associated with growth events and decline with synaptic stabilization (23, 24), we interpret the LTE-related decline in F1/GAP-43 mRNA

FIG. 3. Decreased F1/GAP-43 gene expression in subfield CA3 of the hippocampus related to 3-day synaptic enhancement in animals receiving unilateral HFS or LFS. (A) HFS caused a decrease in F1/GAP-43 mRNA expression (*, $P < 0.05$, $n = 5$; paired t test). No decrease occurred after LFS. (B) The alterations in F1/GAP-43 gene expression were inversely related to synaptic enhancement. Animals were implanted with electrodes bilaterally but were stimulated only unilaterally. LFC, LFS controls.

expression as reflecting synaptic stabilization processes which follow initial growth or modification. This initial growth shortly after the first HFS could be accompanied by increased F1/GAP-43 expression or by posttranslational modifications of the existing protein without increased expression.

Demonstration of a statistical correlation between levels of immediate early gene mRNA expression and degree of synaptic enhancement described here have been hinted at by recent reports. However, in those studies altered gene expression after LTE has provided only sparse quantitative analyses, rarely reporting mean values for mRNA alterations or statistical comparisons with the magnitude of LTE (25–28). In fact, prior studies have not demonstrated a relation between alterations in gene expression and the extent of synaptic enhancement (25, 29). In contrast, the changes in mRNA expression reported here are highly consistent and statistically significant. Since F1/GAP-43 and γ -PKC are target genes, not immediate-early genes, perhaps their altered expression after LTE represents the net correlated consequence of the combinatorial effects of individual uncorrelated factors.

The alterations in F1/GAP-43 mRNA in CA3 may be initiated by monosynaptic perforant path input to CA3 cells or due to a retrograde signal from CA1 after direct monosynaptic activation or disynaptic activation occurring via the CA3 Schaffer collaterals. In either case, if the reduction of mRNA levels in CA3 results in a similar reduction of protein, there should be a decrease in F1/GAP-43 protein in the Schaffer collateral terminals, since F1/GAP-43 is predominantly localized to presynaptic terminals. This predicts that increased synaptic efficacy would be stabilized in the CA3-CA1 synapses. Indeed, after HFS of the perforant path, subsequent stimulation results in increased polysynaptic responses in CA1 (6).

A subset of animals which received LFS and a subset which received HFS without expressing LTE had slight increases in F1/GAP-43 expression at 3 days. We have reported large increases in F1/GAP-43 gene expression in hippocampus after seizures (17) and have recently also observed small increases in F1/GAP-43 and PKC subtype mRNA levels 1 hr after either HFS or LFS in acute, anesthetized preparations (P.J.M. and A.R., unpublished results). Thus, nonspecific stimulation may be responsible for the

slight increases in F1/GAP-43 expression. If the decline in F1/GAP-43 mRNA levels is interpreted as contributing to synaptic stabilization, then perhaps all animals initially have a nonspecific increase, while an active suppression of F1/GAP-43 expression would occur only in those animals destined to express persistent LTE.

The alterations in gene expression observed in the present study could depend either on the induced expression of transcription factors (such as those encoded by immediate-early genes; refs. 25 and 29) or on phosphorylation of existing transcription factors. Since the activity of certain transcription factors is regulated by PKC phosphorylation (30, 31), perhaps increased PKC activity following LTE induction is involved not only in modifying synaptic proteins (2) but also in altering the gene expression of proteins involved in the persistence of LTE.

Of potential relevance in this regard is the strong correlation between LTE-induced alterations in F1/GAP-43 and γ -PKC. The parallel changes in F1/GAP-43 and γ -PKC expression may be due to coordinate regulation through similar gene regulatory elements, possibly involving those transcription factors regulated by phosphorylation. Alternatively, the coordinate regulation could involve a sequence of events in which γ -PKC phosphorylates those transcription factors or mRNA stabilization factors (see below) that regulate F1/GAP-43 expression. For example, F1/GAP-43 mRNA stability may be regulated by phosphorylation of proteins which bind to the 3' end of the mRNA (32). If phosphorylation by γ -PKC were to modify the binding properties of such proteins that regulate mRNA turnover, then alterations in the levels of γ -PKC (regulated perhaps by transcription) would result in parallel alterations in F1/GAP-43 mRNA levels.

To determine the selectivity of the changes observed in mRNA levels after LTE, *in situ* hybridization of other related mRNAs was also examined. Neurogranin has homology with F1/GAP-43 (33), but in contrast to the predominately presynaptic localization of F1/GAP-43, it is a postsynaptic PKC substrate (34). MAP-2 is a structural protein (35) whose mRNA is also located postsynaptically in dendrites (10) and whose phosphorylation state in the visual cortex is modified by visual experience (9). Since changes in the number of receptors or conductance of individual receptors have been suggested as mechanisms for enhanced synaptic transmission

(12), we investigated the B-flip glutamate receptor subunit, which has a greater ion conductance than the alternatively spliced "flop" form (12). None of these mRNAs exhibited alterations related to LTE persistence.

We have previously reported increases in F1/GAP-43 phosphorylation after 3 days that correlate positively with synaptic enhancement (3). Direct comparisons of phosphorylation results from that study with the alterations in mRNA expression reported here cannot be made, since gene expression was measured in cells restricted to the hippocampus whereas the phosphorylation study assayed protein F1/GAP-43 from presynaptic terminals of both intrinsic and extrinsic origin. The present findings, however, make it less likely that increased F1/GAP-43 phosphorylation 3 days after LTE induction is due to increased protein levels, since the F1/GAP-43 protein level is regulated by its mRNA level (e.g., ref. 24).

We describe here a change in gene expression 3 days after LTE of F1/GAP-43 and PKC subtypes, whose phosphorylation and enzyme activity, respectively, are significantly increased within 1 hr after LTE induction (1–4). This suggests the generalization that enduring changes in synaptic efficacy or memory formation may initially require phosphorylation of select proteins, followed by changes in gene expression of the very same proteins that initially participate in the post-translational modification.

Note Added in Proof. The recent report of altered LTP in a PKC- γ null mutant mouse provides further evidence for a role of this PKC subtype in synaptic plasticity (37).

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