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Imaging of Memory-Specific Changes in the Distribution of Protein Kinase C in the Hippocampus

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Activation of protein kinase C (PKC) can mimic the biophysical effects of associative learning on neurons. Furthermore, classical conditioning of the rabbit nictitating membrane (a form of associative learning) produces translocation of PKC activity from the cytosolic to the membrane compartments of the CA1 region of the hippocampus. Evidence is provided here for a significant change in the amount and distribution of PKC within the CA1 cell field of the rabbit hippocampus that is specific to learning. This change is seen at 1 day after learning as focal increments of [^{3}H]phorbol-12,13-dibutyrate binding to PKC in computer-generated images produced from coronal autoradiographs of rabbit brain. In addition, 3 days after learning, the autoradiographs suggest a redistribution of PKC within CA1 from the cell soma to the dendrites.

PROTEIN KINASE C (PKC) IS ENriched within the pyramidal cells of the hippocampus (*1, 2*) and may be a critical intracellular second messenger in associative learning (*3, 4*). There is an increase of membrane-associated PKC in the CA1 region of hippocampi after classical conditioning in rabbits (*5*), an effect that has been attributed to translocation of the enzyme (*6, 7*). To determine how the observed conditioning-induced translocation of PKC activity is distributed among and within neurons of the hippocampus, we have employed in vitro quantitative autoradiography (*8*). [^{3}H]-Phorbol-12,13-dibutyrate ([^{3}H]PDBU), a highly specific radioligand for membrane-associated PKC, was used to determine the spatial localization of brain PKC in classically conditioned rabbits both 1 day and 3 days after the behavioral experience.

In the initial set of experiments, rabbits were randomly assigned to one of three groups. Group C (conditioned) received a 400-ms 1-kHz tone (CS), which terminated at the same time as a 100-ms periorbital electric shock (UCS), for 80 trials per day for 3 days. Group UP (unpaired) animals also received an identical number of CSs and UCSs per day over 3 days, but in an explicitly unpaired fashion. Group N (naïve) remained in their home cages for 3 days. Conditioned and unconditioned responses were measured as a retraction of the nictitating membrane as had been described (*5, 9*). All group C animals showed reliable conditioned extension of the nictitating membrane (>90% conditioned responses) by the end of the third day of conditioning. After the animals were killed on day 4 of the experiment, their brains were processed for quantitative autoradiography by standard methods (*10*).

The use of [^{3}H]PDBU as a quantitative autoradiographic radioligand for PKC has been well established (*11, 12*). This phorbol ester has a much higher affinity for PKC associated with the membrane than for the cytosolic enzyme and is relatively soluble in the aqueous phase (*13*). The relatively low lipid solubility of PDBU compared with other phorbol esters results in low nonspecific binding in autoradiographic assays (*11, 12*). Film autoradiograms were analyzed on a computerized imaging system (Imaging Research) based on a charge-coupled device; the system quantified tissue radioactivity from measured autoradiographic optical densities.

We validated the autoradiography assay by means of an exogenous pharmacological challenge. Muscarinic agonists such as carbachol activate and increase membrane-associated PKC (*6, 7*). To assess the extent to which activation and translocation is manifest by an increase in autoradiographically determined [^{3}H]PDBU binding, we prepared from naïve animals hippocampal slices (400 μm thick) of the type used in the "electrophysiological slice" preparation (*14*). The slices were then exposed to either 10 m*M* carbachol or artificial cerebrospinal fluid (ACSF) for 20 min and then processed for autoradiography (*10*). Carbachol-incubated slices showed a 19 ± 3% increase in [^{3}H]-PDBU binding over controls ($P < 0.01$ Student's t test, $n = 20$ slices each group). The [^{3}H]PDBU binding assay revealed a carbachol-induced increase of membrane-associated PKC and therefore provided evidence that the technique itself did not translocate all the PKC and that amounts of PKC associated with membranes as measured by other methods (*6, 7*) were comparable to amounts measured with our assay. This result demonstrates the sensitivity of the [^{3}H]-PDBU autoradiographic method to the exogenous activation or membrane-association of PKC, or both.

Representative quantitative autoradiographic images from each of the three groups in the initial experiment are shown in Fig. 1. For each coronal section through the dorsal hippocampus, a region of interest (ROI) was produced (in a manner that was blind with respect to the experimental groups; see legend to Fig. 1) around the CA1 cell field. Multiple sections from each animal were analyzed for specific binding in this ROI, and these values for each section were averaged for each animal. Group C animals showed a 49 ± 7% and 43 ± 13% increase in [^{3}H]PDBU specific binding over the UP and N groups, respectively, representing statistically significant differences [$F = 8.716$, $P < 0.01$, one-way analysis of variance (ANOVA), $n = 5$ in each group]. There was no significant difference between the binding values for UP and N groups. In all sections, nonspecific binding was less than 8%. These quantitative images demonstrate statistically significant focal increases in [^{3}H]PDBU binding within the selected ROI (which included the stratum pyrimidale and stratum oriens of CA1 cell fields)

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from conditioned rabbits. Increases were also seen less consistently in CA3 but these trends did not reach statistical significance. The observation of such a trend is not unexpected, since the CA3 cells are presynaptic to those of CA1 and receive similar extrinsic innervation (*15*).

Transept-line image analysis of digitized autoradiograms shows maximal specific binding in the stratum pyrimidale and, to a lesser extent, stratum oriens in group C animals as seen in the representative image transept lines (Fig. 2A). Within-subject variability was assessed by overlaying transept-line profiles from multiple tissue sections from the same animal (Fig. 2B). In all cases, the characteristic pattern of [^{3}H]PDBU binding as represented by these multiple transept lines through the hippocampus was maintained throughout the tissue sections analyzed.

In order to test whether the effect of learning on PKC was persistent, two additional groups of animals (group 3-C and 3-UP; $n = 5$ for each group) were added. Groups 3-C and 3-UP were treated identically to group C and UP, respectively, except that their brains were examined 3 days instead of 1 day after behavioral treatment as described above for groups C and UP. Autoradiographic analysis of image transept-line profiles was performed in an identical manner for group 3-C and C and showed relatively more binding at the stratum oriens (apical dendrites of CA1 pyramidal cells) concomittant with less binding at the stratum pyrimidale relative to the dendrites (Fig. 2C). Within-subject transept-line variability was assessed as for groups C and UP and is shown in Fig. 2D.

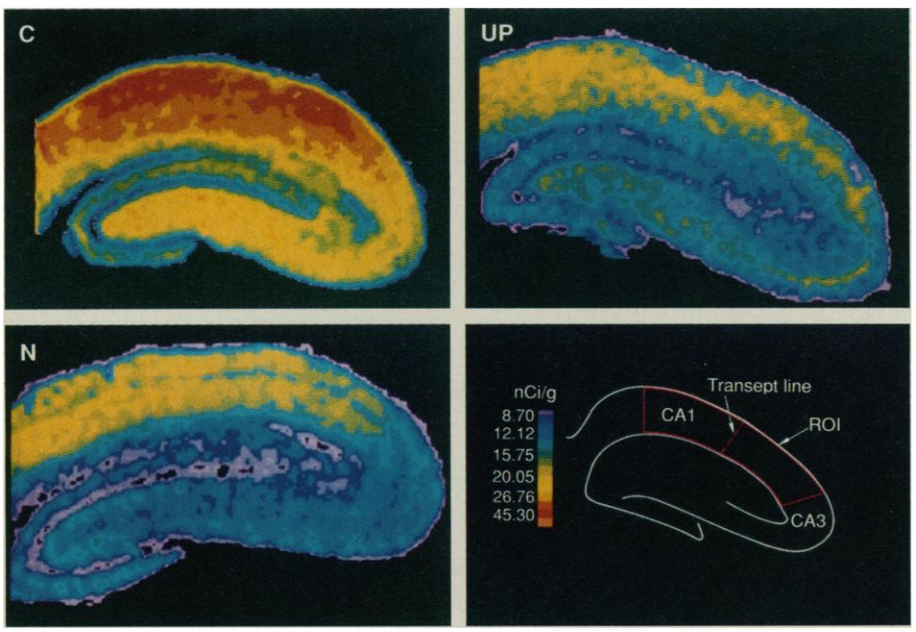

Fig. 1. Computer-generated pseudocolor images of PKC distribution in hippocampi from three groups: C, UP, and N. In all sections the region of interest (ROI) was determined (in a manner blind to the experimental groups), dorsally, by the border between the alveus and the corpus callosum; medially, by a line perpendicular to the tangent line at the dorsal border of the genu of the CA1 region; laterally, as a perpendicular to the tangent line at the border of CA1 and CA3; and ventrally, by the border of hippocampal fissure (see lower right). These sections ran from 3 mm to 5 mm posterior to bregma at approximately 100-μm intervals (*25*). The ROI included the full width of the dendritic arborizations of the pyramidal cells including the strata moleculare, lacunosum, radiatum, pyrimidale, and oriens to the alveus. This trapezoidal ROI was superimposed with the use of a computer-controlled mouse on an image of the autoradiogram of each section as displayed on the image processing system. The number of sections available for analysis per animal averaged 15 and was never less than 5. Both left and right hippocampi were pooled because preliminary data had suggested that there were no differences in [^{3}H]PDBU binding between the two. The number of total slices analyzed per animal was not significantly different from one group to another as analyzed by one-way ANOVA. A random sampling of 12 sections from all of those used showed a variability of approximately 6% in the number of pixels included in each ROI. Each ROI value thus represented the average bound [^{3}H]PDBU contained within approximately 2500 pixels. This value was then stored by the computer in a log file along with an identifier, which allowed the software to compute grand ROI averages over all of the hippocampal sections sampled for each animal (this grand mean then represented the average radioactivity value in nanocuries per gram of wet weight of brain tissue of some 75,000 pixels per animal—each pixel represents approximately 75 μm^2 of tissue area). These grand mean values for each animal were then used to compare groups. ($F = 8.716$, $P < 0.01$, one-way ANOVA, $n = 5$ in each group). The conditioned group (C) had significantly more [^{3}H]PDBU binding within the selected ROI than the UP or N groups. The color bar in the lower right provides quantitative calibration for all three images. Although the increase is dramatic and manifests itself as focal islands of nonuniformly spread [^{3}H]PDBU binding within the CA1 cell field, the more subtle differences in binding between the stratum pyrimidale and stratum oriens are not demonstrable in these images and were analyzed with the more sensitive transept-line analysis in Figs. 2 and 3.

Transept lines were compared between animals by normalizing to the point of inflection corresponding to the stratum pyrimidale (SP) and a point 7 pixels to the left of the border between the SP and the stratum oriens (SO) thus forming an SP:SO ratio (Fig. 3). Group C animals had significantly higher ratios than control groups whereas group 3-C animals had significantly lower ratios. Thus, the significant decrease in the SP:SO ratio indicated that there was a new distribution of the label within the hippocampus 3 days after conditioning compared with the distribution 1 day after conditioning. Taken together with the overall increase in [^{3}H]PDBU binding seen in CA1 for the group C animals, the transept-line profile results suggest that, after an initial increase in membrane-associated PKC in the cell soma, the enzyme migrated to the dendritic compartment of the CA1 pyramidal cells, with a resultant decrease in the SP:SO ratio. This highly significant, conditioning-specific change in the distribution of PKC (Fig. 3) as measured by the SP:SO ratio of [^{3}H]PDBU binding represents a change in the pattern of PKC distribution that is dependent on retention time of the learned behavior.

Our data are consistent with previous autoradiographic studies that have shown relatively low amounts of [^{3}H]PDBU binding localized to pyramidal cell perikaria in hippocampus for subjects naïve to any behavioral manipulation (*11, 12*). Immunohistochemical studies have shown PKC immunoreactivity within pyramidal cell bodies (*11, 12*), and it is possible that in our classically studied conditioned animals the increase in soma-localized enzyme (as assayed by [^{3}H]PDBU binding) may represent de novo enzyme synthesis. Newly synthesized enzyme might subsequently be transported to postsynaptic sites of modification.

We have shown a learning-specific change in the amount and spatial distribution of membrane-associated PKC within the CA1 field of hippocampus that depends on retention time. One day after conditioning there was an increase of membrane-associated PKC over the soma and dendritic region. Three days after conditioning the dendritic region had significantly more label relative to the soma than either conditioned animals after 1 day (group C) or controls. Although our results presented here support the hypothesis of a conditioning-dependent trans-

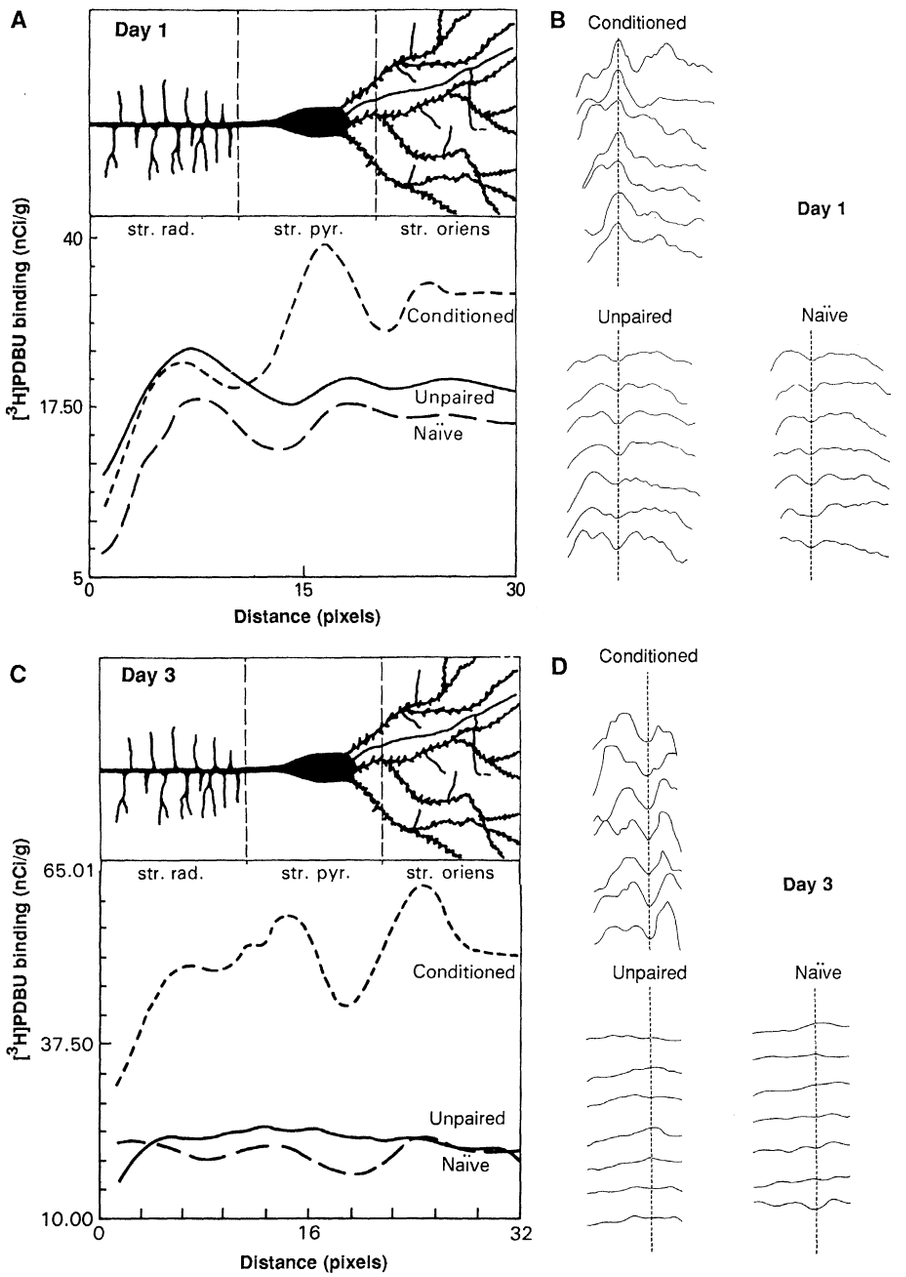

Fig. 2. (**A**) Transept-line profiles across CA1 from three representative rabbits 1 day after conditioning: groups C, UP, and N. The transept line (see Fig. 1, lower left) was determined for each section analyzed as that line segment midway between the medial and lateral borders of the ROI (see Fig. 1, lower right) and perpendicular to the tangent line at the dorsal edge of the ROI. For the transept-line analysis, hippocampal images were enlarged by a factor of 2 and subjected to a low-frequency 3 × 3 convolution matrix filter operation in order to smooth the data. The transept line was then placed, by means of a computer-controlled mouse, at a point midway along the lateral-medial extent of the ROI described in detail above. Each transept line provided approximately 40 points of specific binding data along the length of the transept. The abscissa shows distance in pixels along the transept line. The cartoon of the CA1 pyramidal cell is added to aid in the interpretation of the graphs. Although it is not drawn to scale, the longitudinal extent of it (along the abscissa) is an accurate representation of the pyramidal neuron dendritic fields, which correspond to the graphs (str., stratum; rad., radiatum; pyr., pyrimidale). (**B**) For 1-day retention rabbits, the within-subject transept-line profiles (across multiple neighboring tissue sections) were similar. The within-subject similarity in these profiles is typical for all sections analyzed. (**C**) Representative transept lines across the CA1 region produced as in (A) for 3-day retention animals (groups 3-C and 3-UP) and naïve groups. [^{3}H]PDBU binding tended to be a higher for transept-line profiles from group 3-C than for those from group C but did not reach statistical significance. For between-group statisical comparisons see Fig. 3. (**D**) Within-subject variability: 3-day retention rabbits. Transept lines are plotted from multiple tissue sections within the same animal as in (C). The naïve animal transept lines in this figure are from a different individual than the one plotted in (B).

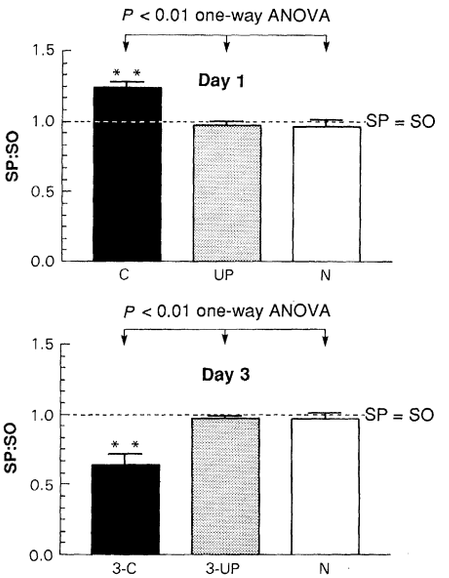

Fig. 3. (**Top**) Between-group comparisons of transept lines for 1-day retention animals. Normalization was accomplished with a ratio (SP/SO) obtained for each slice. Maximum (or minimum) binding values along the transept-line portion within the cell body layer were divided by a binding value for the stratum oriens (measured on the transept line 7 pixels from the border between the stratum pyrimidale and stratum oriens layers). When no obvious maximum or minimum values were apparent in the stratum pyrimidale layer (as was usual for the controls) the average transept value within the stratum pyrimidale layer provided the numerator of the ratio. This SP:SO ratio was then compared between groups by one-way ANOVA. The bar graphs demonstrate a statistically significant change (35 ± 3% and 43 ± 5% increase in SP/SO in group C compared with group UP and N, respectively, $P < 0.01$, one-way ANOVA, $n = 5$ animals, 75 sections per group) in the pattern of PKC distribution within the hippocampus as a function of learning. (**Bottom**) Between group comparisons of transept lines for 3-day retention animals and control groups (3-UP and N). The bar graphs show a statistically significant decrease in the SP/SO ratio (35 ± 2% and 30 ± 5%, respectively, compared with groups 3-UP and N, $P < 0.01$, one-way ANOVA, $n = 5$ animals, 75 sections per group) calculated as for 1-day retention animals.

location of PKC activity to the membrane, they would also be consistent with a selective increase in the type I isozyme of PKC, which is preferentially associated with the cell membrane and is highly enriched in CA1 (*16*).

The [^{3}H]PDBU binding assay does not distinguish between labeling of CA1 pyramidal cells and labeling of nonneuronal cells in close proximity. Nor is the nature of the translocation event yet well understood in molecular terms. However, increases in [^{3}H]PDBU binding that were observed within the stratum oriens may prove to be localized specifically to the dendrites of individual CA1 pyramidal cells.

Our observations suggest that changes in

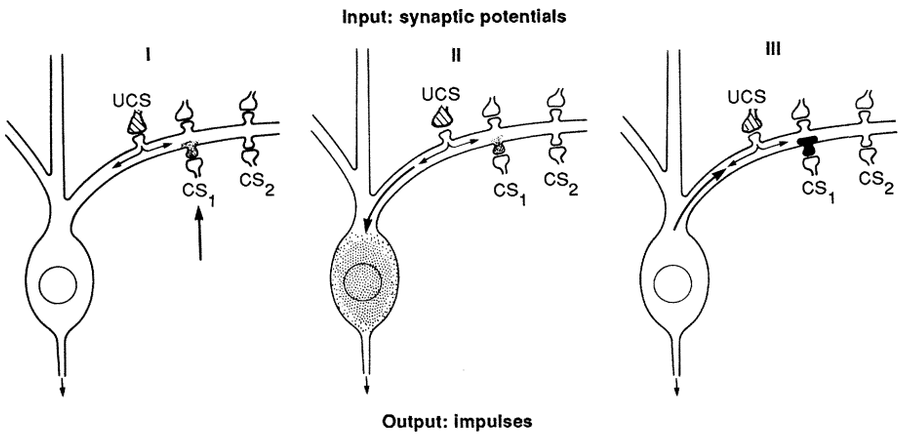

Fig. 4. Hypothetical model demonstrating activation of PKC in soma and dendrites. I, initial activation of both CS_1 and UCS results in local activation of PKC at the postsynaptic spine. II, After the initial activation of CS_1 and UCS (and dependent on whether the two activating imputs are paired), a "retrosignal" causes an increase in PKC synthesis within the cell body (1-day retention condition). III, Finally, in the last stage of "consolidation," specifically targeted, newly synthesized proteins (including PKC) make their way back to the initially activated postsynaptic dendritic region, resulting in a long-term change in the biophysical characteristics of the spine in response to new inputs via the CS_1 input pathway. CS_2, input from an unpaired CS.

PKC localization may subserve some of the learning-specific electrophysiological and biochemical changes seen in the hippocampus. In vivo studies have demonstrated increased unit activity (*17*) in the hippocampus after nictitating membrane conditioning in the rabbit. Short-term in vitro increases in synaptic membrane glutamate receptor binding have been observed in the hippocampus 1 hour after conditioning in the rabbit (*18*), whereas longer term in vitro studies have shown decreased afterhyperpolarization (*19*) and increased synaptic summation in pyramidal cells of CA1 24 hours after conditioning (*20*). Lesion and behavioral studies indicate that these changes in hippocampus may contribute to storage of information about the spatial and temporal context of the learned response rather than the conditioned response itself (*21*). Other brain areas (for example, the cerebellar cortex H VI and associated deep nuclei) more directly implicated in storing or expressing the conditioned response show differences in membrane-associated PKC (*22*).

The mystery of why a single discrete conditioning paradigm affects so many CA1 neurons has been raised by the present and past findings (*17–19*). We speculate that afferent input localized to specific dendritic compartments first induces changes within those postsynaptic compartments. These initial postsynaptic changes send biochemical or electrical signals (or both) to the cell body. At the cell body, in a second phase of memory formation (Fig. 4) cellular changes are triggered that in turn increase transport of crucial molecules that localize or have localized effects in the initially active dendritic compartments. Thus, during consolidation of memory formation many cell bodies would be involved, but during more permanent memory retention only specific dendritic compartments could be altered. This hypothetical sequence is supported by the data from group 3-C (Figs. 2 and 3) showing conditioning-specific, long-lasting dendritic changes in [^{3}H]PDBU binding and may be analogous to sequential involvement of spatially separated cellular compartments during chromatolysis (*23*).

In conclusion, our experiments show a conditioning-specific increase in hippocampal membrane-bound PKC concomitant with a change in its hippocampal distribution. PKC activation results in a reduction in Ca^{2+}-dependent K^+ currents in rabbit paramidal cells from CA1 (*3*) and within type B photoreceptors of the mollusk *Hermissenda crassicornis* (*3*). Similar reductions have been observed to persist for days after classical conditioning in rabbit (*19*) and *Hermissenda* (*24*). Taken together with the present findings, these results are consistent with the hypothesis that PKC activation and translocation in the hippocampal formation play important roles in the storage and biophysical expression of associative memory.

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