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Classical conditioning induces long-term translocation of protein kinase C in rabbit hippocampal CA1 cells

(memory storage/membrane-associated protein kinase)

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ABSTRACT **The role of the Ca^{2+}/phospholipid-dependent, diacylglycerol-activated enzyme protein kinase C (PKC) in rabbit eyelid conditioning was examined. PKC was partially purified from the CA1 region of hippocampal slices from naive, pseudoconditioned, and conditioned rabbits 24 hr after the rabbits were well conditioned. Crude membrane and cytosol fractions were prepared. In conditioned rabbits, significantly more PKC activity (63.3%) was associated with the membrane fraction (and significantly less with the cytosol fraction) compared to naive (42.0%) and pseudoconditioned (44.7%) animals. These differences in distribution of enzyme activity were paralleled by differences in stimulation of enzyme activity by Ca^{2+}, phospholipid, and diacylglycerol. There were no between-group differences in basal protein kinase activity. These results suggest that there is a long-term translocation of PKC from cytosol to membrane as a result of conditioning. Autoradiographic binding of radioactive phorbol 12,13-dibutyrate to PKC demonstrated that almost all specific binding was in the stratum radiatum, a region containing the proximal apical dendrites of CA1 pyramidal neurons. Therefore, this may be the site of the conditioning-specific PKC translocation, a locus well-suited to underlie the biophysical effects of conditioning.**

Recent work in our laboratory has established that classical conditioning of the nictitating membrane/eyelid response causes a biophysical record similar to that of *Hermissenda* classical conditioning—both records involving persistent reductions of a Ca^{2+}-dependent outward K^+ current, $I_{K(Ca)}$ (1–4). A long-lasting decrease in $I_{K(Ca)}$ was measured as a decrease in the late afterhyperpolarization (AHP) following impulses of hippocampal CA1 cells (1, 2). In addition, it was found that conditioning induces an enhancement in the summation of postsynaptic potentials (5). Both the AHP reduction and the enhanced postsynaptic potential summation can be mimicked by bath application of phorbol esters, a class of pharmacological agents that activate Ca^{2+}/phospholipid-dependent protein kinase C (PKC) (5–7). Similarly, in *Hermissenda*, injection of purified PKC into type B photoreceptors reduces I_A (an early, rapidly inactivated outward K^+ current) and $I_{K(Ca)}$, but only when paired with a "calcium load" (8). We therefore examined the role of PKC activation as a possible biochemical mechanism underlying the biophysical changes induced by classical conditioning. We now report a molecular record of nictitating membrane/eyelid conditioning: a long-lasting (24 hr), conditioning-specific translocation of PKC activity from the cytosol to the membrane compartment of CA1 cells isolated from the hippocampal slice. This finding is novel because the translocation is of long duration, is learning-specific, and is intrinsic to a population of vertebrate neurons known to undergo biophysical alterations as a result of this type of learning. In addition, the present finding provides a possible point of convergence between mechanisms of learning and hippocampal long-term potentiation (LTP), which is also associated with translocation of PKC activity 1 hr after LTP induction (9).

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METHODS

For the present study, male albino rabbits weighing 1–2 kg were classically conditioned to blink to a tone by pairing a 400-msec, 1-kHz, 85-decibel tone with a coterminating 150-msec, 1- to 2-mA periorbital shock. Rabbits received 80 trials per day (60-sec mean variable intertrial interval) for 3 days, after which they were well trained (greater than 80% conditioned responding on blank test trials). Control animals were either pseudoconditioned (tone and shock randomly but explicitly unpaired with a 30-sec mean variable intertrial interval) or naive. On the day after the last training session, slices of the left hippocampus were prepared according to standard procedures (10). The slices were allowed to equilibrate for 0.5 hr in artificial cerebrospinal fluid. The CA1 region of four slices from each animal was microdissected and pooled (Fig. 1). The slices were homogenized in 250 μl of buffer containing 50 mM Pipes (pH 7.5), 10 mM EGTA (to minimize artifactual redistribution of PKC due to calcium), 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, leupeptin at 20 μg/ml, and aprotinin at 20 μg/ml. The homogenate was centrifuged at 100,000 × *g* for 1 hr at 4°C to separate particulate and soluble proteins. The supernatant was designated as the crude cytosol fraction. The particulate fraction was further homogenized in another 250 μl of homogenization buffer containing 0.3% Triton X-100 and allowed to stand on ice for 1 hr in order to extract membrane-bound PKC. The solubilized membranes were centrifuged again at 100,000 × *g* for 1 hr at 4°C and the supernatant thus obtained was designated as the crude membrane fraction. PKC activity was then partially purified by threonine-Sepharose column chromatography by a slight modification of the procedure of Kikkawa *et al.* (11). Samples (250 μl) were applied to L-threonine-Sepharose 4B columns (0.6 cm × 0.5 cm) that were equilibrated with 20 mM Tris·HCl, pH 7.5/0.5 mM EGTA/0.5 mM EDTA/10 mM 2-mercaptoethanol. After the column was washed with 2.5 ml of equilibration buffer and then 0.5 ml of 0.2 M NaCl in equilibration buffer, bound PKC activity was eluted with 1 ml of 1.0 M NaCl in equilibration buffer. The column fraction thus eluted contained protein kinase that was dependent upon Ca^{2+}, diacylglycerol, and phosphatidylserine (PtdSer) for full ac-

Abbreviations: AHP, afterhyperpolarization; ANOVA, analysis of variance; CS, conditioned stimulus; $I_{K(Ca)}$, Ca^{2+}-dependent K^+ current; LTP, long-term potentiation; PBt_2, phorbol 12,13-dibutyrate; PKC, protein kinase C; PtdSer, phosphatidylserine; US, unconditioned stimulus.

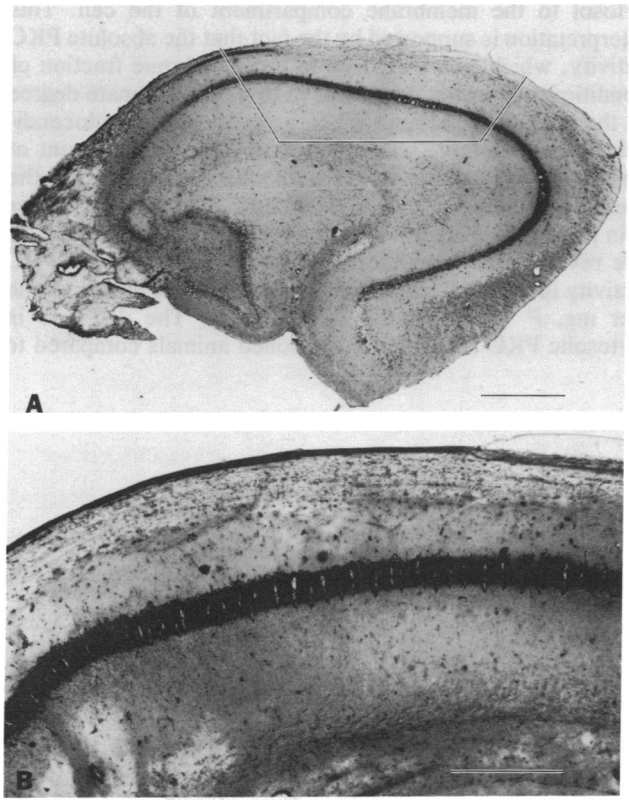

FIG. 1. (*A*) Hematoxylin-stained section (300-μm thick) of dorsal hippocampus. Lines demarcate region dissected for biochemical analysis. Darkly stained band consists of CA1 cell bodies. (Bar = 5 mm.) (*B*) Enlargement of dissected region indicated in *A*. (Bar = 1 mm.)

tivity (data not shown). PKC activity was determined by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into histone H1 in the absence or presence of PKC activators. The assay mixture (250 μl) contained 20 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 50 μg of histone H1, 10 μM ATP (2 μCi of [γ-^{32}P]ATP; 1 μCi = 37 kBq), 12.5 μg of PtdSer, 1 μg of diolein (dioleoylglycerol), 0.5 mM $CaCl_2$, and 50 μl of enzyme source. The mixture was incubated for 3 min at 30°C and the reaction was terminated by placing 25-μl aliquots of reaction mixture onto phosphocellulose strips (1 cm × 2 cm) that were immediately immersed in 75 mM phosphate buffer (pH 2.1) and rinsed for 1 hr. After three more such rinses (2 min each), the strips were air-dried and radioactivity was determined by liquid scintillation counting. Basal activity was determined by replacing Ca^{2+}, PtdSer, and diolein with 0.5 mM EGTA. All biochemical analyses were performed "blind."

For autoradiographic assay of binding of radioactive phorbol 12,13-dibutyrate ([^{3}H]PBt_2) to PKC, hippocampal slices (300–700 μm) were prefixed (2.5% glutaraldehyde in 0.05 M cacodylate at pH 7.4; 10 min), incubated with 2.5 nM [^{3}H]PBt_2 either alone or in combination with 2.5 μM nonradioactive PBt_2 (to determine the extent of nonspecific labeling) for 60 min, washed with buffer (30 min), further fixed (60 min), and embedded in 5% agarose. Vibratome sections (10–20 μm) were cut and mounted on gelatin/chrome alum-coated slides, coated with Ilford KD5 emulsion, exposed for 4–6 weeks, and developed. Silver grains were counted over the strata oriens and radiatum and the pyramidal-cell-body layer.

RESULTS

Basal protein kinase levels (i.e., in the absence of Ca^{2+}, PtdSer, and diacylglycerol) were virtually identical in the three groups for both membrane and cytosolic compartments (Table 1). Addition of Ca^{2+} alone did not stimulate protein kinase activity. We also found no difference in total PKC activity (membrane plus cytosol) among conditioned (2.15 ± 0.43 nmol per min per mg of protein, n = 10), pseudoconditioned (2.42 ± 1.23, n = 9), and naive rabbits (2.50 ± 0.62, n = 10; $P > 0.25$, one-way ANOVA, all values mean ± SEM) (Fig. 2 *Upper*). However, there was a highly significant difference between the conditioned group and the pseudoconditioned and naive groups with respect to the intracellular distribution of PKC activity (Fig. 2 *Lower*). In conditioned rabbits, 63.3 ± 4.1% of the enzyme activity was associated with the membrane fraction, whereas the membrane fractions from pseudoconditioned and naive rabbits contained only 42.0 ± 5.0% and 44.7 ± 5.1%, respectively ($P < 0.01$, one-way ANOVA). Post-hoc pairwise comparisons indicated that the percentage of membrane-bound PKC activity was significantly greater in conditioned animals than in naive ($P < 0.05$) or pseudoconditioned animals ($P < 0.01$,

Table 1. Basal protein kinase levels are not affected by conditioning

Fraction	Protein kinase activity, nmol per min per mg		
	Conditioned	Pseudoconditioned	Naive
Membrane	2.58 ± 0.75	2.65 ± 0.56	2.32 ± 0.46
Cytosol	3.15 ± 1.02	3.70 ± 1.10	3.47 ± 1.18

All values are means ± SEM. Values for fractions from pseudoconditioned or naive rabbits do not differ significantly from values for the corresponding fractions from conditioned rabbits [one-way analysis of variance (ANOVA)].

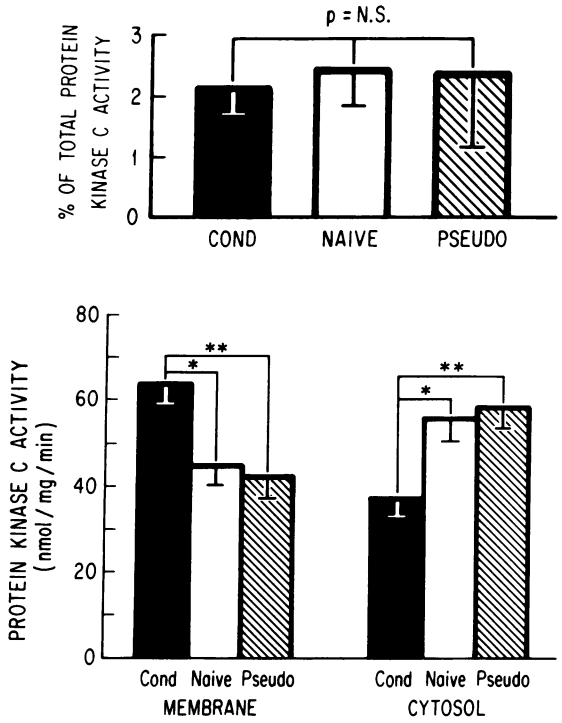

FIG. 2. Conditioning causes a redistribution of intracellular PKC with no effect on total PKC activity. (*Upper*) Total PKC activity was calculated as the membrane-bound specific PKC activity plus the cytosolic specific PKC activity divided by 2. Differences between the means were tested by a one-way ANOVA. All values are means with only the negative standard error included. N.S., not significant. (*Lower*) The effects of the various treatments on the percentage of PKC activity associated with each cellular compartment was assessed by a one-way ANOVA with post-hoc Neuman–Keuls multiple pairwise comparisons. *, $P < 0.05$; **, $P < 0.01$; n = 10 (conditioned), n = 9 (pseudoconditioned), n = 10 (naive).

Neuman–Keuls multiple pairwise comparison). Naive and pseudoconditioned groups did not differ significantly from one another (Fig. 2 *Lower*). Conversely, the cytosolic fraction from conditioned animals possessed a lower amount of activity (36.7%) compared to pseudoconditioned (58.0%) or naive (55.3%) control animals. These data were supported by the finding that Ca^{2+}/PtdSer/diacylglycerol gave 46% more stimulation of membrane PKC activity from conditioned animals (244 ± 22.2%) relative to controls (198 ± 9.8%, $P < 0.05$, one-tailed t test) and 33% less stimulation of cytosolic PKC from conditioned animals (188 ± 21.5%) relative to controls (221 ± 9.8%). These findings suggest that there was a translocation of PKC activity from the cytosol to the membrane compartment of the cell. This interpretation is supported by the fact that the absolute PKC activity, which was increased in the membrane fraction of conditioned animals, was reduced to a proportionate degree in the cytosolic fraction. While in naive and pseudoconditioned animals there was a significantly greater amount of absolute PKC activity in the cytosolic fraction versus the membrane fraction (0.78 ± 0.39 and 1.88 ± 0.96 nmol per min per mg of protein, respectively), in conditioned animals the reverse was true: there was significantly greater PKC activity in the membrane fraction (0.81 ± 0.38 nmol per min per mg, $P < 0.01$, one-way ANOVA). The decrease in cytosolic PKC activity in conditioned animals compared to

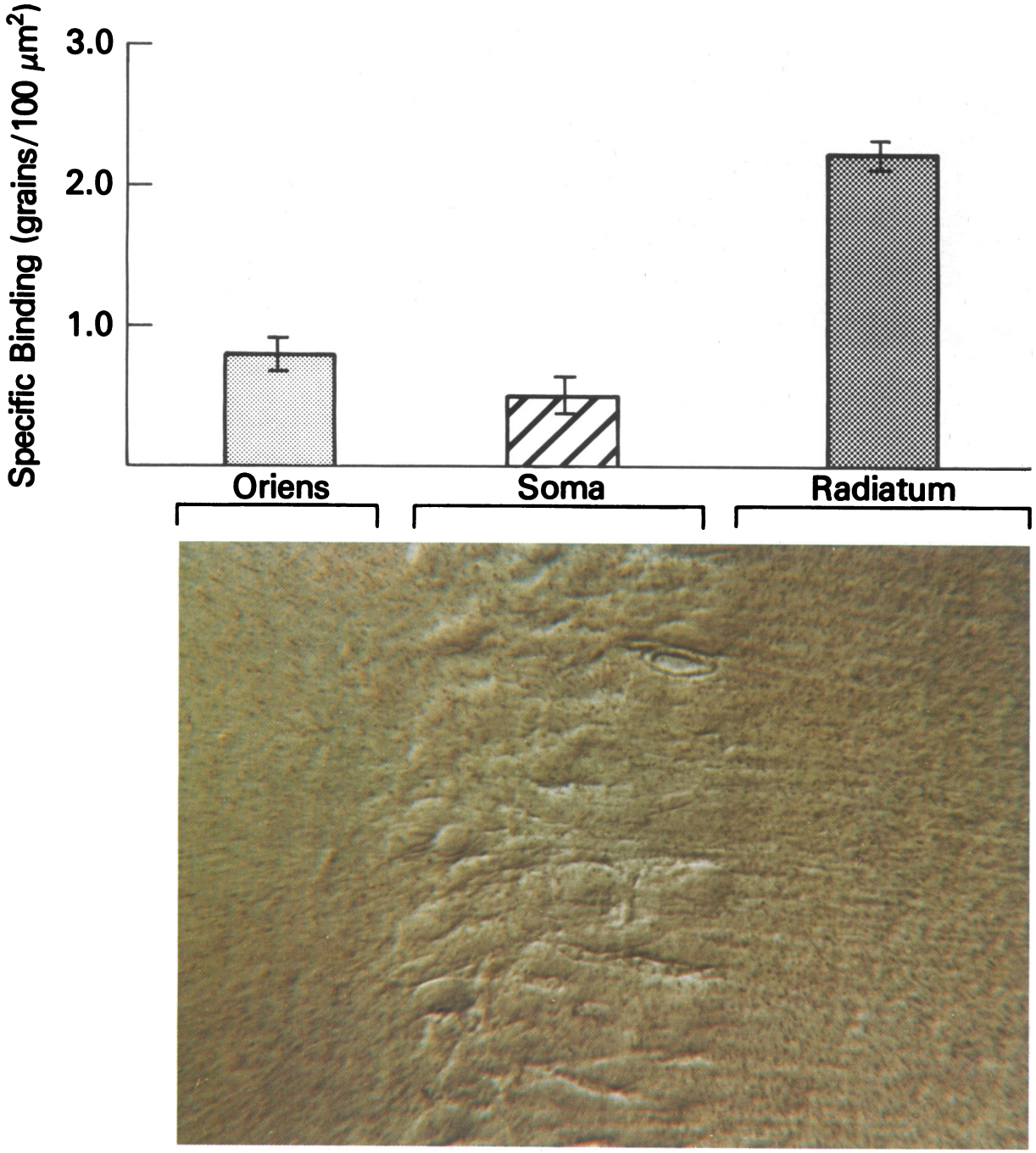

FIG. 3. PKC is localized in stratum radiatum. PKC was autoradiographically visualized after incubation of hippocampal slices from naive animals with [^{3}H]PBt_2. Specific binding reflects the number of grains visualized after incubation with 2.5 nM [^{3}H]PBt_2 minus that observed after incubation of adjacent slices with 2.5 nM [^{3}H]PBt_2 plus 2.5 μM unlabeled PBt_2. Error bars represent standard deviations ($n = 10$).

controls (≈1.5 nmol per min per mg) was mirrored by a corresponding increase in membrane-bound PKC activity (≈0.8 nmol per min per mg). The increase in membrane-bound PKC activity was only half of the decrease in cytosolic activity because there was a 2:1 ratio of solubilized membrane protein to cytosolic protein.

Autoradiographic localization of PKC was achieved after incubation of hippocampal slices with [^{3}H]PBt$_2$. Total (specific plus nonspecific) binding was observed in the stratum radiatum, the stratum oriens, and the CA1 pyramidal-cell layer (Fig. 3). Specific binding was determined by subtracting label measurements obtained after incubation with 2.5 nM [^{3}H]PBt$_2$ plus a 1000-fold excess of unlabeled PBt$_2$ (see *Methods*) from those obtained after incubation of adjacent sections with [^{3}H]PBt$_2$ alone. Whereas 15% of the binding in the stratum oriens and 10% in the cell-body layer was specific, 50% of the binding in the stratum radiatum was specific (Fig. 3). Therefore, specific binding sites for PBt$_2$ are concentrated in the stratum radiatum (the region containing the CA1 apical dendrites). Binding assays using 30 nM [^{3}H]PBt$_2$ (12) revealed that in naive animals, the PKC distribution is virtually identical to that obtained in naive and pseudoconditioned animals by enzymatic activity assay of PKC purified from cytosolic and membrane fractions. Therefore, the enzyme assay appears to provide a valid indication of PKC distribution.

DISCUSSION

Our results show that temporal pairing of tone and shock results in a long-term association of PKC with the membrane compartment in hippocampal CA1 neurons. This association may occur via some stable covalent modification of the enzyme or, alternatively, as a result of a chronic increase in diacylglycerol turnover causing a greater rate of PKC translocation. The high concentration of EGTA present during the tissue fractionation procedure ensured that no artifactual association of PKC with the membrane occurred. In addition, the fact that high EGTA did not release the enzyme from the membrane suggests that conditioning caused the PKC to become an integral membrane protein.

The possible cellular locus of this translocation was determined by autoradiographic visualization of PKC by [^{3}H]PBt$_2$ binding. This technique revealed that in CA1, almost all specific binding of [^{3}H]PBt$_2$ occurs in the stratum radiatum, the region of CA1 containing the apical dendrites. This could account for the biophysical effects of conditioning—i.e., an enhancement of postsynaptic potential summation (due to the dendritic location of PKC) and a reduction in the AHP (due to the apical dendrites' proximity to the CA1 cell-body layer). Recent experiments using this technique have revealed a 40% increase in silver grains over the CA1 apical dendritic region in conditioned versus pseudoconditioned CA1 pyramidal cells (13).

Activation of PKC occurs when diacylglycerol is generated by the signal-dependent breakdown of inositolphospholipids by phospholipase C (14). Activation appears to involve a transfer of the enzyme to a phospholipid environment (i.e., the plasma membrane) where it can be fully activated in the presence of low Ca^{2+} concentrations (15). The translocation, and hence activation, of PKC is synergistically affected by phorbol esters (non-metabolizable mimics of diacylglycerol) together with Ca^{2+} (16).

In invertebrates, PKC activation can cause increased inward Ca^{2+} current (17) as well as reduced outward K^+ currents (18, 19). In mammals, PKC activation causes a reduction in the late AHP in hippocampal CA1 cells (6, 7) presumed to be mediated by $I_{K(Ca)}$. In our laboratory, we have found that classical conditioning of the nictitating membrane/eyelid response reduces the late AHP (presumably $I_{K(Ca)}$) (1, 2). More recently, we have found that conditioning increases the summation of postsynaptic potentials (elicited by stimulation of the Schaffer collateral fibers) (5). This increase in synaptic summation, which was correlated with AHP reduction, can also be mimicked by bath application of phorbol ester (5). The results reported here and in previous studies strongly suggest that PKC activation could mediate the learning-induced alterations in membrane currents observed in hippocampal CA1 cells 24 hr after learning and that this activation is a result of a long-term (24 hr) translocation of PKC. We speculate that this occurs via a synergistic activation of diacylglycerol production and hence PKC activity [conditioned-stimulus (CS) pathway] and a depolarization-induced Ca^{2+} influx [unconditioned-stimulus (US) pathway]. Ca^{2+} influx may affect Ca^{2+}/calmodulin-dependent kinase(s) [which is known to be concentrated in postsynaptic densities of vertebrate neurons (20)] as well as the PKC pathway. In *Hermissenda*, iontophoretic injection of Ca^{2+}/calmodulin-dependent protein kinase type II into type B cells caused enhanced reduction (21) of the same two K^+ currents reduced by conditioning (3, 4). Furthermore, bath application of phorbol ester also reduced these two K^+ currents only following voltage-clamp conditions that caused prolonged activation of a voltage-dependent Ca^{2+} influx (18).

Activation dependent on both diacylglycerol and Ca^{2+} influx might render PKC no longer susceptible to whatever processes that normally detach it from the membrane, such as proteolysis (22) or autophosphorylation (23). Synergy between Ca^{2+}-mediated and PKC-mediated events for full biological response has been observed in other systems, including those involved in platelet aggregation (24) and aldosterone secretion (25). This putative chain of events may also underlie other forms of plasticity, such as associative LTP (26). Nonassociative LTP induced by stimulation of the perforant path has been shown to result in a translocation of PKC activity from the cytosol to the membrane in dentate gyrus 1 hr after the LTP treatment (9), although the translocation has not been localized to a particular population of neurons.

The biochemical changes observed in the present study and the biophysical changes observed in other studies are intrinsic to the hippocampus. The intrinsic nature of these changes suggests that they serve an actual storage role in eyelid conditioning. Other observations functionally implicate the hippocampus in eyelid conditioning, although not in the generation of simple motor responses. Thus, it has been established that hippocampal lesions do not abolish eyelid conditioning generated by the stimulus conditions used here (simple delay conditioning) (27), but lesion studies have demonstrated that in this paradigm the hippocampus is essential for discrimination reversal (28), blocking (29), sensory preconditioning (30), latent inhibition (31), and appropriate stimulus generalization (32). Furthermore, scopalamine retards delay conditioning only when the hippocampus is present (33), suggesting a modulatory role for the hippocampus during acquisition. These results implicate the hippocampus in the learning of higher-order information regarding the CS–US relationship rather than the learned motor program itself. Such an interpretation is consistent with the view that learning of even discrete stimulus associations includes the storage of various types of information at various loci distributed within the mammalian brain.

An important question raised by this and related studies concerns the number of CA1 cells apparently altered by conditioning. In order to produce a measurable conditioning-specific translocation of PKC, a significant percentage of the total population of cells had to have been affected. Similarly, *in vivo* recordings have demonstrated that 62% of pyramidal cells increase their firing rate in response to the CS after

conditioning (34). Disterhoft *et al.* (1) showed that 48% of CA1 pyramidal cells exhibited markedly reduced AHP responses after conditioning. LoTurco *et al.* (5) showed that over 50% of CA1 cells from conditioned animals exhibited an enhanced summation of postsynaptic potentials elicited by Schaffer collateral stimulation. Why are so many cells affected? And how is specificity preserved for encoding other associations?

One possible explanation concerns the salience of the training experience for the animal. Because of minimal stimulation during maintenance conditions and subsequent exposure to hundreds of CS–US pairings, the conditioning paradigm may have assumed overriding influence on the animal and consequently affected a large number of CA1 cells. Alternatively, a substantial proportion of the CA1 population might be affected by many different associations. In this case, specificity for each association could arise out of a particular distribution of dendritic alterations within a particular combination of CA1 cells. Although the combinations for different associations would share common CA1 cells, no two combinations would be identical (see refs. 35 and 39). By combining phorbol ester binding and pharmacologic lesion techniques, evidence for a dendritic locus of PKC in CA1 cells has in fact been provided (ref. 36 and the present study). Further, the findings of LoTurco *et al.* (5) are consistent with a dendritic locus for conditioning-specific biophysical changes. Thus, conditioning-specific translocation of PKC within dendritic compartments could account for the synaptic alterations as well as the correlated AHP changes previously found with conditioning and allow for specific encoding of many distinct associations.

A final possible basis for the large number of CA1 cells affected by conditioning might be due to a relatively transient period of information storage in the hippocampus for each CS–US association. The hippocampus could be activated in a general manner for every CS–US association during a phase of consolidation (on the order of days) and then no longer be involved in long-term retention (weeks or longer). Indeed, some clinical evidence is consistent with a consolidation rather than a retention role for the hippocampus in memory formation (37).

The present study suggests a molecular mechanism by which the hippocampus may exert its unique influence on information storage. This mechanism may also contribute to classical conditioning of *Hermissenda*, for which a conditioning-specific alteration in the phosphorylation of a M_r 20,000 protein substrate of PKC (as well as Ca^{2+}/calmodulin-dependent kinase) was found (38). Although there have been many hypotheses for long-term biochemical substrates of vertebrate associative learning, the present study provides a strong link between associatively induced behavioral change, intrinsic biochemical change, and corresponding changes of cellular physiology.

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