Learning-Induced Activation of Protein Kinase C

A Molecular Memory Trace

Barry Bank,* Joseph J. LoTurco, and Daniel L. Alkon

Section on Neural Systems, Laboratory of Molecular and Cellular Neurobiology, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda MD 20892

Contents

*Author to whom all correspondence and reprint requests should be addressed.

Introduction

A molecular substrate for associative learning should be uniquely activated by converging sensory/physiological signals (e.g., a conditioned and unconditioned stimulus). That is, when activated by convergent signals, the substrate should undergo in the presence of either signal alone. Furthermore, this convergent persistent activation of a critical molecular substrate should have physiologic consequences (within specific neurons) that express and, thereby, behaviorally demonstrate, storage of an associative memory. Here, we will present evidence obtained from both molluscan and mammalian preparations indicating that the diacylglycerol (DG) activated, calcium/phospholipid-dependent enzyme, protein kinase C (PKC), could serve as a molecular substrate of associative memory.

Protein kinase C is a multifunctional enzyme whose activity is dependent on the presence of three factors: calcium, phospholipid, and diacylglycerol (DG) (*see* Nishizuka, 1984). Activation appears to involve a transfer of the enzyme to a phospholipid environment (i.e., the plasma membrane), where it is fully activated in the presence of DG and low calcium concentrations. DG is gathered in the plasma membrane by enzymatic (i.e., phospholipase C) breakdown of polyphosphoinositides (Berridge, 1984). The translocation of PKC to the plasma membrane, and hence its activation, is synergistically enhanced by a concomitant activation of PKC by both calcium and phorbol esters (nonhydrolyzable analogs of DG) (Wolf et al., 1985).

A ubiquitous role for PKC in higher brain function and neuronal modifiability has been proposed in the past (Nishizuka, 1984: Routtenberg et al., 1985). In the following review we will describe the experimental results that shed light on the role of PKC translocation and activation in the formation and maintenance of associative memories. Our experimental approach has been to identify learning-specific alterations in the biochemical and biophysical characteristics of identified neurons and to subsequently determine the relationship between these alterations. We have observed startling similarities between mechanisms of learning-related modifiability in rabbit hippocampal CA1 neurons and *Hermissenda* B photoreceptors. These cross-species similarities suggest that PKC activation is a crucial associative learning step that has been preserved through the course of evolution.

The Behavioral Paradigm

Classical conditioning, a prototypical form of associative learning, is an ideal behavioral paradigm for the biological analysis of elementary learning processes (Gormezano et al., 1985). Essentially, when a neutral stimulus such as tone or light (the conditioned stimulus—CS) is paired with a stronger response-eliciting stimulus such as an electric pulse or rotation (the unconditioned stimulus—US), the neutral stimulus will come to elicit the same behavioral response as the strong stimulus. This learned response is the conditioned response or CR.

The associative learning paradigm employed for *Hermissenda* involves inhibition of photoaxis (Alkon, 1974; Crow and Alkon, 1978). Classical or Pavlovian conditioning has been demonstrated for a specific muscular response component of phototactic movement (Lederhendler and Alkon, 1986). Light (the CS) evokes a slight lengthening of the foot muscle (the single organ of locomotion), whereas rotation (the US) elicits a brisk reflexive contraction of the same muscle. Following repeated pairing of light and rotation, light alone will elicit food contraction (Lederhendler and Alkon, 1986), an entirely new response.

For rabbit eyeblink conditioning, a common procedure is to pair a tone (the CS) with a mild electric pulse (the US) applied to the face. This US elicits a reflexive eyeblink and a simultaneous sweep of the nictitating membrane (the unconditioned response—UR). After sufficient

numbers of pairings, the tone itself will come to elicit the eyeblink and nictitating membrane response.

There are many similarities between *Hermissenda* and rabbit classical conditioning. The conditioned stimuli are both nonaversive sensory inputs, whereas the unconditioned stimuli are aversive in nature. The conditioned responses in each preparation are muscular reflexes (contraction of the foot muscle in *Hermissenda* and contraction of the ocular and facial muscles in rabbits) (Lederhendler and Alkon 1986; McCormick et al., 1982; Cegavske and Thompson, 1976). More important, the conditioned stimuli come to evoke new behavioral responses that resemble the UR, that is, there is UR-CR transfer. The conditioning paradigms employed for *Hermissenda* and rabbit are characterized by many critical defining features, which are hallmarks of Pavlovian conditioning. These features include: dependence on the CS-US interval, stimulus specificity, and long-term retention (Gormezano et al., 1983; Lederhendler and Alkon, 1986). Additional features shared by rabbit and *Hermissenda* conditioning are: contingency learning (Kehoe, 1979; Farley, 1987), extinction (Gormezano et al., 1962; Richards and Farley 1984), and superior conditioning for forward pairing of CS and US (Smith et al., 1969; Lederhendler and Alkon, 1986), and savings (Crow and Alkon, 1978).

One advantage of employing classical conditioning for the analysis of cellular correlates of learning is that it affords the opportunity for rigorous control procedures. When the CS and US are presented independently (what we refer to as pseudoconditioned or random presentation) animals receive the same number of CSs and USs as conditioned animals, and are exposed to training for the same length of time, but do not learn to blink to the tone, in the case of the rabbit, or to contract the foot in response to light, in the case of *Hermissenda*. This procedure controls for any nonassociative effects of training such as habituation, sensitization, stress, fatigue, or handling. In addition, naive controls enable assessment of effects caused by the unpaired procedure. By employing these three groups, possible biophysical or biochemical effects of conditioning can be unequivocally attributed to the association of the CS and US.

Identification of Neurons Involved in Conditioning

Studies have shown that the B cells in *Hermissenda* and the hippocamal CA1 neurons in rabbit are involved in classical conditioning (Alkon, 1984; Berger et al., 1983). In *Hermissenda*, the B photoreceptors are inhibitory to the A photoreceptors, which are in turn excitatory to interneurons and motor neurons that control foot contraction and locomotion (Goh et al., 1984; Lederhendler et al., 1986). During conditioning, increased excitability of the B cells results in a shortening of the foot and a decrease in photoactic behavior—the conditioned response (Alkon, 1984).

The hippocampus' role in rabbit classical conditioning is less well understood than the role of the B cells in *Hermissenda* classical conditioning. From in vivo extracellular unit recordings made during training, it has been well documented that over 50% of CA1 pyramidal cells exhibit an increase in firing frequency that highly correlates with the topography of conditioned eyeblinks (Berger et al., 1983). Ablation of the hippocampus does not disrupt the occurrence of conditioned responses (Schmaltz and Theios, 1972), but ablation of the hippocampus has been shown to alter the latency (Port et al., 1985) and shape (Orr and Berger, 1985) of conditioned responses, and to severely impair performance in complex conditioning tasks (Berger and Orr, 1983; Port and Patterson, 1984; Solomon, 1977; Solomon and Moore, 1975). Furthermore, pharamacological disruption of the hippocampus significantly impairs acquisition of conditioned responses (Solomon et al., 1983), and induction of long-term potentiation in the

hippocampus greatly facilitates the acquisition of conditioned responses (Berger, 1983). Thus, in *Hermissenda,* the B cells are critical to the acquisition and expression of conditioned responses, whereas in rabbit the hippocampus modulates the acquisition and expression of conditioned responses.

Classical Conditioning and PKC Activation

Protein kinase C is a reasonable molecular substrate for classical conditioning, because it is most fully activated by two convergent signal-dependent pathways (calcium and diacylglycerol), which leads to a sustained physiological response. No other protein kinase displays such a unique requirement. In other mammalian cells, calcium and diacylglycerol-signaled systems play an important role in sustaining physiological responses that persist once cytosolic calcium concentrations have returned to basal levels (*see* Alkon and Rasmussen, 1988). From an extensive series of experiments in adrenal glomerulosa cells, Rasmussen and colleagues have proposed that the sustained physiological response (in this case, aldosterone secretion) involves temporal integration of two branches of the calcium messenger system. One branch is mediated by a calmodulin dependent process and the other by a PKC dependent process (Kojima et al., 1984). A strikingly similar synergistic activation of PKC is observed in platelet release reactions (Kaibuchi et al., 1983) and acetylcholine release in guinea pig ileum (Tanaka et al., 1985) among others. This dual activation is reminiscent of the requirements for associative learning: temporally associated CS and US occurrence. This prompted us to speculate that the persistence of biophysical records of classical conditioning are mediated by long-term translocation and, hence, activation of PKC.

We tested this hypothesis by assaying PKC activity in membrane and cytosol compartments of rabbit hippocampal CA1 neurons from conditioned, pseudoconditioned, and naive rabbits. These cells are known to undergo long-term increases in excitability as a consequence of rabbit eyelid conditioning (Berger et al., 1983; Disterhoft et al., 1986; Coulter et al., 1986; LoTurco et al., 1987). Crude cytosolic and membrane fractions were prepared from the CA1 regions microdissected from hippocampal slices. PKC activity was partially purified by threonine sepharose column chromatography. PKC activity was determined by the degree to which it phosphorylated an exogenous PKC substrate (lysine-rich histone). Twenty-four hours after conditioning there was no difference between the three groups with respect to either basal protein kinase activity or total PKC activity (membrane + cytosol). However, there was a highly significant difference among groups with respect to the intracellular distribution of activity of this enzyme. In conditioned rabbits, 63.3% of the enzyme activity was associated with the membrane fraction, whereas pseudoconditioned and naive rabbits contained only 42.0 and 44.7%, respectively. Conversely, the cytosolic fraction from conditioned animals possessed a lower amount of activity (36.7%) compared to pseudoconditioned (58.0%) and naive (55.3%) animals (Bank et al., 1988) (Fig. 1). These findings imply that there was a translocation of PKC activity from the cytosolic to the membrane compartment of the cell as a result of conditioning. This translocation is relatively long lasting. In addition, the association of PKC with the membrane is extremely stable (i.e., it has become an integral membrane protein), since it was not released by high concentrations of the calcium chelator EGTA, suggesting that the enzyme no longer requires calcium for its association with the membrane.

An important question raised by this finding concerns the number of CA1 cells apparently altered by conditioning. In order to measure a conditioning-specific translocation, a significant percentage of the total population of cells had to have been effected. In fact, in vivo record-

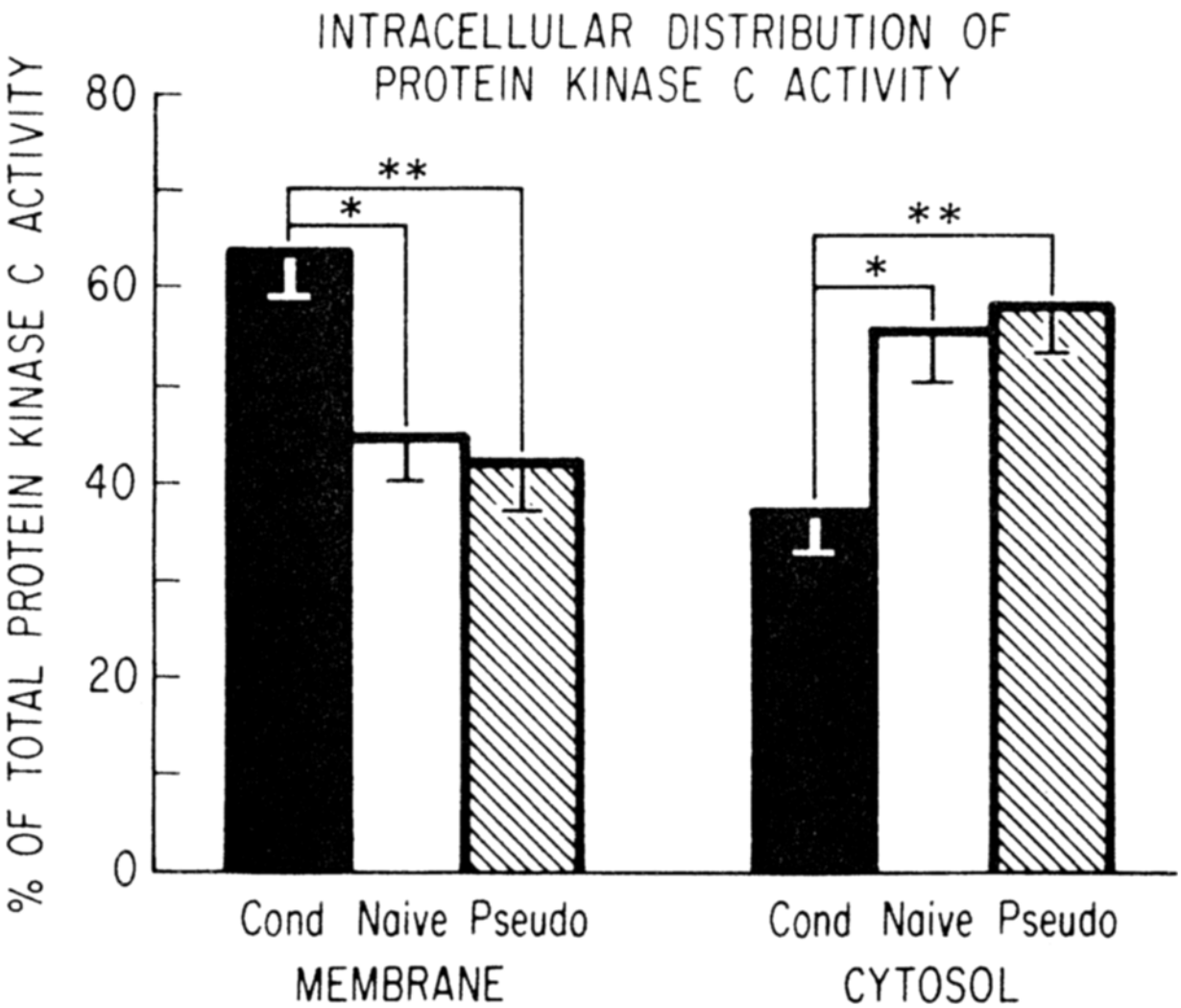

Fig. 1. Translocation of PKC activity from the cytosol to the membrane in area CA1 is induced by classical conditioning (*see* text for further explanation).

ings have demonstrated that 62% of pyramidal cells increase their firing rate in response to the CS after conditioning (Berger et al., 1983). In a system that undergoes massive change, specificity for encoding multiple associations might arise out of a particular ensemble of CA1 cells. Such a hypothesis requires that the changes are localized to dendrites. We have recently begun to determine the cellular locus of the conditioning-specific translocation. Radioactive labeling of PKC with tritiated phorbol ester applied to hippocampal slices suggests that the PKC is particularly concentrated in the stratum radiatum where the proximal dendrites of CA1 pyramidal cells are located (Bank et al., 1988). These results suggest that temporally contiguous stimulus presentations result in the long-term translocation of PKC in specific subcellular regions of hippocampal CA1 neurons. More recently, studies from our laboratory have used [^{3}H] phorbol dibutyrate autoradiography to visualize PKC activation as a result of conditioning (Olds et al., 1988). One day after training, there is increased labeling of the soma and,

to a lesser extent the dendrite layers of the CA1 cell region in the conditioned but not control animals. Three days after conditioning, this soma-dendritic layer distribution is reversed, i.e., now the dendrite layer is preferentially labeled in the conditioned animals (Olds et al., 1988; and, in preparation). In addition to hippocampus, we have studied PKC activation in hemispheric lobule VI (HVI), a structure found to be crucial for the acquisition and retention of the conditioned NMR (Yeo et al., 1985; Lavond et al., 1987). Here, too, PKC redistribution from cytosol to membrane occurs 24 h after conditioning (Bank et al., 1988b). In addition, the amount of redistribution correlates highly with a loss of total cellular PKC content. PKC proteolysis in the membrane compartment is a known consequence of extensive PKC activation (Ballester and Rosen, 1985). This conditioning-induced loss of total cellular PKC was also observed as a trend in the hippocampal CA1 region (Bank et al., 1988a). This proteolytic step in membrane-bound PKC activation may signal the nucleus to regulate long-term synthesis of this enzyme.

We have attempted to mimic the effects of conditioning by exposing hippocampal slices to agents that elicit both depolarization-induced calcium influx (secondary to elevation of external K^+) and increased DG turnover (stimulated by exposure to glutamate). Even up to 1 h (and perhaps longer) after this manipulation, PKC remains translocated to the membrane, suggesting that PKC translocation is not a transient signal-dependent process, but rather a sustained process.

There is also evidence that PKC is involved in classical conditioning in *Hermissenda*. Three days of classical conditioning causes an increase in the in vivo phosphorylation of a 20 KD MW protein in the eyes of conditioned animals relative to unpaired and naive controls (Neary et al., 1981). No other protein bands were similarly affected. Using both in vitro (Neary et al., 1986) and in vivo (Alkon et al., 1988) phosphorylation assays, the 20 KD phosphoprotein has been shown to be a PKC substrate. Moreover, the 20 KD MW phosphoprotein phosphorylated by PKC is a membrane-associated protein (Alkon et al., 1988). This suggests that conditioning in *Hermissenda* causes a long-term activation (and hence translocation) of PKC that leads to increased phosphorylation of specific membrane-bound substrates.

If the conditions of training are partially simulated by depolarizing the *Hermissenda* nervous system for prolonger periods of time (30 min), it is also possible to observe long-term changes (at least 30 min) in the phosphorylation of specific proteins that may be the result of PKC translocation. A cytosolic substrate for PKC, a phosphoprotein with a MW of 23 kD, remains dephosphorylated for at least 0.5 h after depolarization by high K^+ (Naito et al., 1988). This suggests that prolonged depolarization contributes to translocation of PKC, as indicated by the long-term reduction in the phosphorylation of a cytosolic PKC substrate. Thus, these biochemical observations suggest that PKC activation may be an important step in classical conditioning of *Hermissenda* as well as in rabbit.

Physiological Correlates of Associative Memory Are Mimicked by PKC Activation

Biophysical records of classical conditioning have been revealed in *Hermissenda* B photoreceptors (Alkon, 1984) and rabbit hippocampal CA1 pyramidal neurons (Disterhoft et al., 1986; Coulter et al., 1987; LoTurco et al., 1988). By comparing cells isolated from animals that were classically conditioned, with cells from animals that received explicitly unpaired stimulus presentations (random or pseudoconditioned) or cells from naive animals, it has been determined that *Hermissenda* B cells and rabbit CA1 hippo-

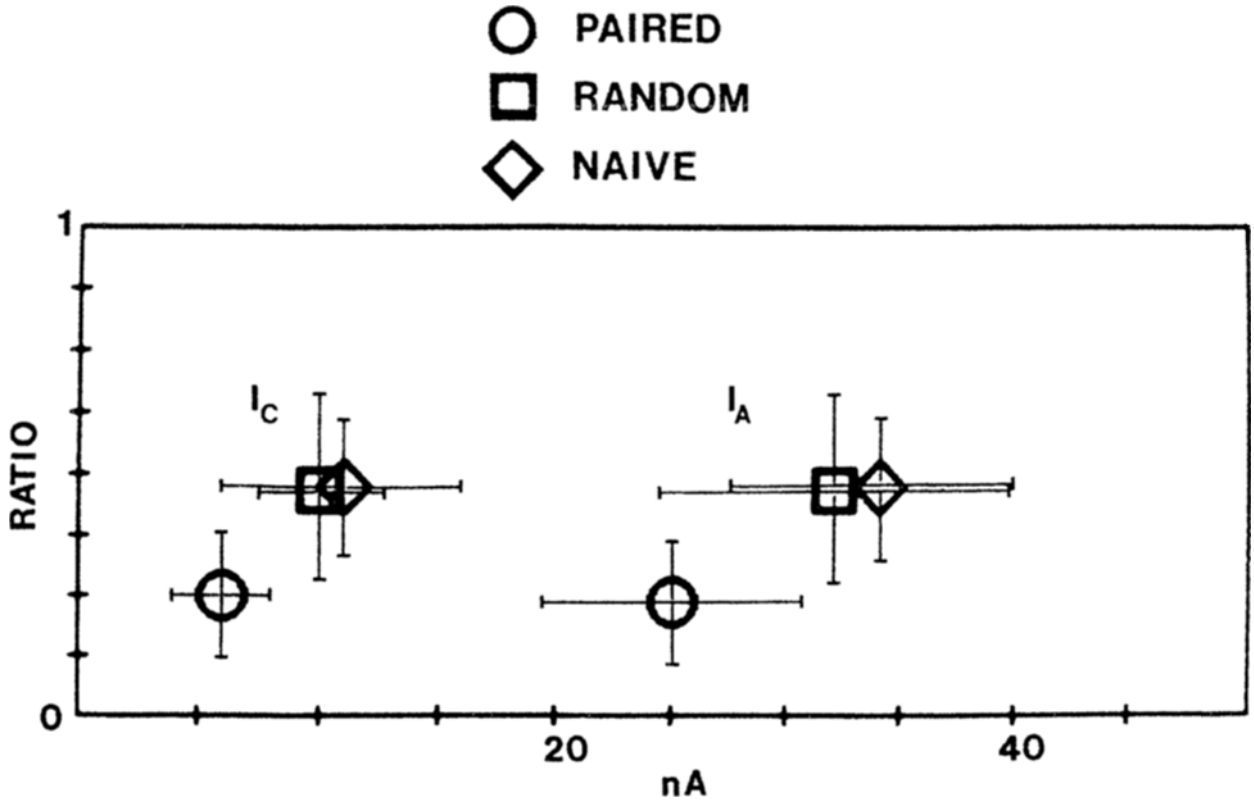

Fig. 2. Biophysical records of classical conditioning in *Hermissenda* type B photoreceptors. The ordinate is the behavioral supression ratio (the measure of learned phototactic inhibition) for *Hermissenda* that received paired, random, or no training (mean ±SEM). The abscissa is the amplitudes of I_A and $I_{K(Ca^{++})}$ recorded from voltage-clamped B photoreceptors isolated from *Hermissenda* in each of the three behavioral conditions (mean ±SEM). Note, in animals that received paired training, the amplitude of I_A and $I_{K(Ca^{++})}$ is reduced.

campal pyramidal cells become more excitable after classical conditioning. This increased excitability is a result of changes in at least two distinct physiological properties in each cell type. In *Hermissenda* B cells two potassium currents are reduced (Alkon, 1984) and in rabbit hippocampus the AHP (after-hyperpolarization potential) is reduced (Disterhoft et al., 1986; Coulter et al., in press) and the summation of postsynaptic potentials (psps) is enhanced (LoTurco et al., 1988).

To establish a casual relationship between the biochemical and biophysical records of conditioning it is necessary to demonstrate that artificial activation of the biochemical pathway that is activated with conditioning (i.e., PKC activation) induces that same biophysical alterations as conditioning. Indeed, activation of PKC induces conditioning-specific physiological characteristics in both *Hermissenda* B cells and hippocampal CA1 neurons. Thus, a single biochemical agent (PKC) may orchestrate the distinct learning-specific changes in both B cells and CA1 neurons.

The first biophysical records of associative learning in any organism were identified in *Hermissenda* B cells (Alkon, 1984). Early and more recent studies all indicate that classical conditioning induces persistent (>24 h) correlated reductions in the transient (I_A) and calcium-dependent ($I_{K(Ca^{++})}$) potassium current in the B photoreceptors (Fig. 2) (Alkon et al., 1985). In addition to the amplitudes of the currents, the kinetics also change with conditioning. More-

over, since induction of membrane changes in single photoreceptors were found to cause behavior resembling associative learning (Farley et al., 1983), and fully account for increased activity in motor neurons responsible for generation of the conditioned response, the decrease in potassium current in B cells is a likely antecedent of the associatively learned foot contraction in response to light.

More recently, I_A and $I_{K(Ca^{++})}$ have been shown to be reduced by bath applied phorbol esters and injection of purified PKC (Alkon et al., 1986; Alkon et al., 1988). Bath application of either phorbol esters, synthetic analogs of diacylglycerol (OAG) (Alkon et al., 1986) or intracellular injection of purified PKC (Alkon et al., in press) were effective in decreasing I_A and $I_{K(Ca^{++})}$ only if combined with intracellular calcium loads (Fig. 3).

Two learning-specific correlates have been identified in rabbit hippocampal CA1 pyramidal cells: a reduction in the AHP and in enhancement in the summation of psps (Fig. 4, *see* p. 10). A major current underlying the AHP (particularly as measured by Disterhoft et al., 1986 and Coulter et al., 1986) is a calcium dependent potassium current (Hotson and Prince, 1980; Lancaster and Adams, 1986). Thus, a physiological parallel between learning-specific changes in *Hermissenda* and hippocampus is a reduction in calcium dependent potassium currents. The synaptic changes that has been identified is an increase in the summation of synaptic potentials. This enhanced summation appears to involve NMDA (*N*-methyl-D-aspartate) receptors, since it is blocked by APV (2-amino-5-phosphonovaleric acid) and may be linked to a decrease in a dendritic potassium conductance (LoTurco et al., 1987). Moreover, the presence of a significant correlation between the reduced AHP and enhanced psp summation only in cells from conditioned animals suggests that these two learning-specific correlates share a common origin that could be PKC activation. PKC activation by 250 n*M* phorbol ester (DPBA), like classical conditioning, markedly decreases the AHP and enhances the summation of psps (Fig. 3). This represents a second parallel between the physiological records of learning in CA1 pyramidal neurons and *Hermissenda* B cells: They are induced not only by conditioning but also by PKC activation.

The biophysical records of classical conditioning in *Hermissenda* and rabbit represent coordinated learning-specific alterations in membrane properties. The coordination of these properties seems an important feature of the physiological records in *Hermissenda* and rabbit, and thus, it becomes important to identify possible common mechanisms. One attractive model that accounts for such coordination is a common biochemical pathway inducing coordinated learning-specific changes. Since PKC activation in *Hermissenda* B cells reduces both I_A and $I_{K(Ca^{++})}$, and in rabbit hippocampal CA1 neurons reduces the AHP and enhances psp summation (Fig. 3), persistent activation of PKC (Bank et al., 1988) is a reasonable common pathway for the induction and coordination of learning-specific alterations in *Hermissenda* photoreceptors and rabbit hippocampus.

Protein Kinase C and Other Forms of Neuronal Modifiability

Protein kinase C has been implicated in long-term potentiation (LTP). LTP is a long-term increase in the effectiveness of synaptic transmission that is induced by high frequency stimulation of many limbic and cortical pathways (*see* Teyler and Discenna, 1987). LTP induced in the perforant path results in PKC translocation in the dentate gyrus for at least 1 h after the LTP treatment (Akers et al., 1986). There is also a corresponding increase in the phosphorylation of the membrane-bound phosphoprotein F1 (or B-50) after LTP, which lasts up to 3 d (Lovinger et al., 1985). Both the degree of PKC translocation and F1 phosphorylation coorelate highly with the degree of

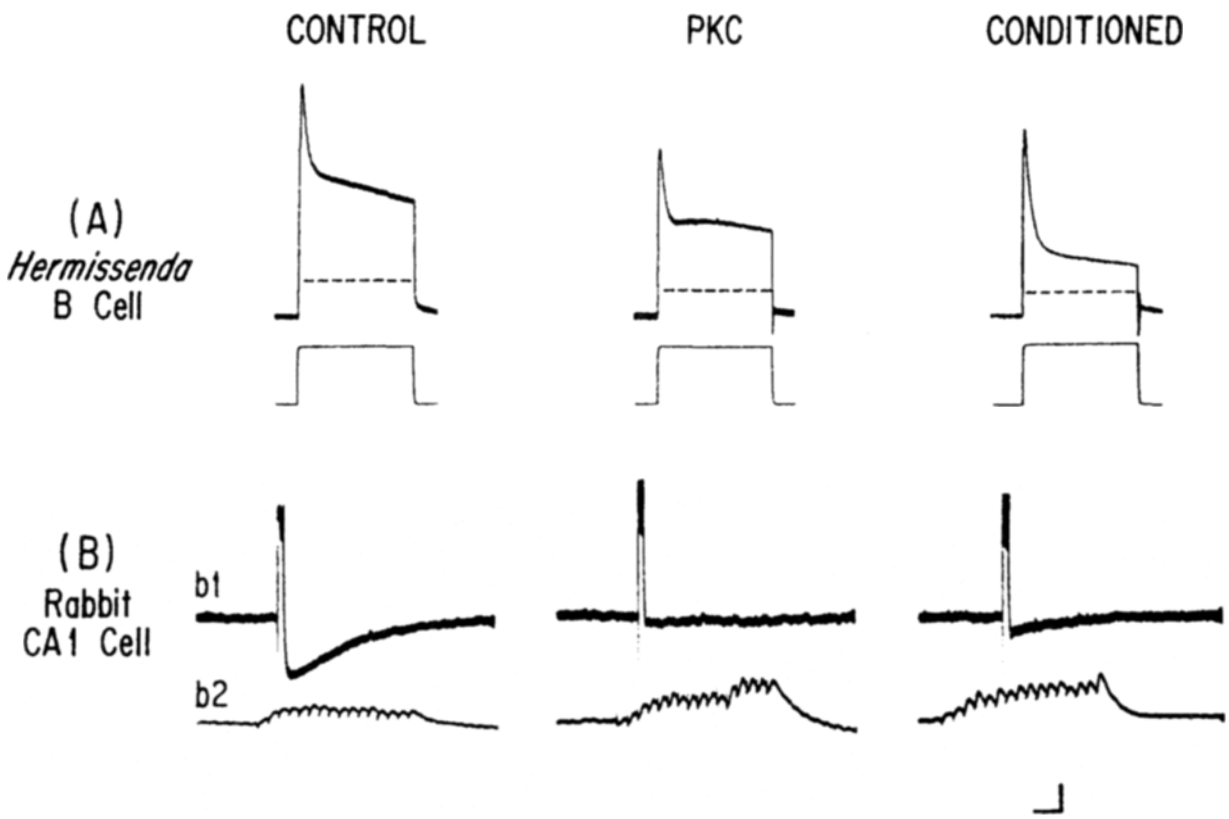

Fig. 3. Activation of PKC in rabbit CA1 cells and *Hermissenda* B cells induces effects similar to those observed after conditioning. **A:** Voltage-clamp records from *Hermissenda* B cells. PKC refers to injection of purified PKC in the presence of phorbol esters (500 n*M*) (calibration: 600 msec, 50 mV). PKC activation together with a Ca^{++} load reduces both I_A and $I_{K(Ca^{++})}$. Reduction in these currents has been shown to be casually related to classical conditioning in *Hermissenda*. **B:** Intracellular records obtained from CA1 pyramidal cells maintained in vitro. PKC activation refers to bath application of 250 n*M* phorbol esters. b1) AHPs following four action potentials (calibration: 500 msec, 5 mV). b2) synaptic potentials elicited by 50 Hz, 300 msec stimulation (calibration: 50 msec, 10 mV). Both reduction in AHP and enhancement of psp summation are induced by either PKC activation or classical conditioning.

potentiation observed (Akers et al., 1986; Routtenberg and Lovinger, 1985).

Activation of PKC by bath application of phorbol esters in hippocampal slices cause potentiation of synaptic transmission of the Schaffer collateral to CA1 neuron synapse (Malenka et al., 1986). This potentiation prevents further LTP produced by electrically induced synaptic input (Malenka et al., 1986). Another form of LTP, "associative" LTP, is produced by pairing a weak synaptic input with a strong synaptic input. Wigstrom and colleagues have shown that the sufficient conditions for inducing associative LTP are a single synaptic volley briefly preceding or concurrent with strong postsynaptic depolarization (Gustaffson et al., 1987). This "associative" LTP effect appear to involve NMDA receptors, since it is blocked by APV (Gustaffson et al., 1987). These LTP findings are consistent with our model of the cellular basis of conditioning. For LTP lasting 1–2 h, glutamate-induced PKC translocation, coupled with depolarization-induced calcium influx, could result in long-lasting association of PKC with the plasma membrane and, thereby, contribute to increased synaptic responsiveness.

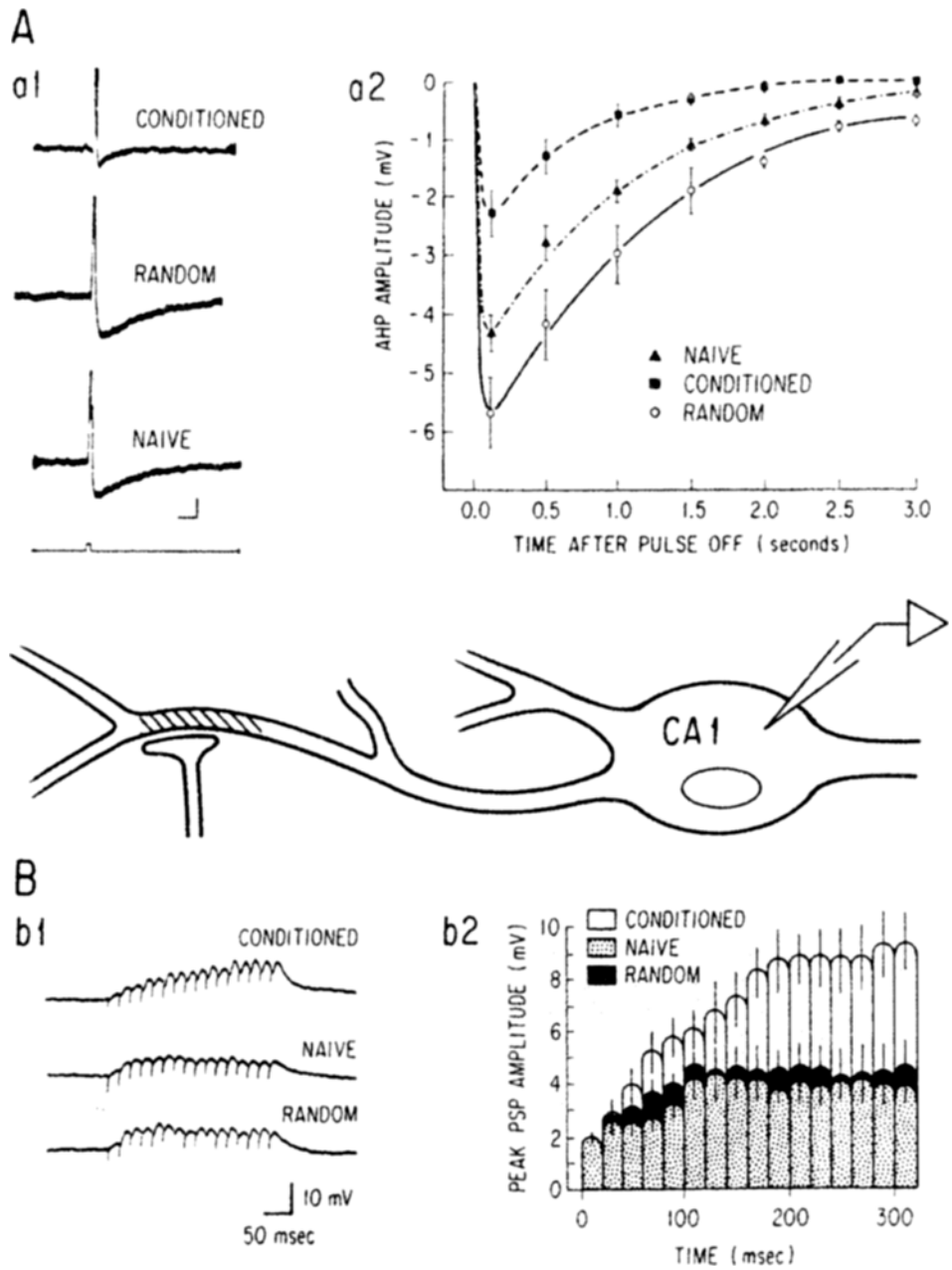

Fig. 4. Biophysical correlates of classical conditioning recorded from rabbit hippocampal CA1 neurons. **A:** The AHP is reduced by classical conditioning: representative recordings or hippicampal CA1 cells from conditioned, random (i. e., unpaired), or naive animals (calibration, 5 mV and 0.5 s); and, averages and sems of AHPs recorded from cells of animals in the three behavioral conditions ($n = 23$). Measurements were made at isochronal points. **B:** Synaptic potentials are enhanced by classical conditioning: b1) Potentials to 300 of 50 Hz stimulation. b2) Profile of the peak psp amplitudes across 300 msec of 50 hz stimulation in cells from conditioned random and naive animals (each, $n = 15$, mean $\pm$SEM).

A Model for the Biochemical Basis of Associative Learning

In many biological systems, the activation of PKC alone does not always elicit a physiological response. Usually, PKC activation has to be accompanied by increases in intracellular calcium for a full biological response (*see* Nishizuka, 1984). The fact that bath application of phorbol ester or synthetic analogs of DG only reduces I_A and $I_{K(Ca^{++})}$ if paired with a calcium load (Alkon et al., 1986) suggests a similar requirement in *Hermissenda* B cells. In the presence of DG or phorbol ester, basal levels of calcium are sufficient to fully activate PKC (Wolf et al., 1985), a calcium load would not be necessary to reduce K^+ currents. Therefore, the high levels of intracellular calcium (Alkon et al., 1986) required to reduce I_A and $I_{K(Ca^{++})}$ may be doing something in addition to directly activating PKC. This raises the possibility that there is a synergistic interaction between a calcium-dependent event and a DG/phorbol ester-dependent activation of PKC to yield complete reduction of I_A and $I_{K(Ca^{++})}$. The synergistic requirement between calcium and PKC-mediated events for full biological activity has been observed in other systems, such as platelet aggregation (Kaibuchi et al., 1983) and aldosterone secretion (Kojima et al., 1984; 1985). This pathway is particularly well suited for mediating associatively induced alterations in the biophysical characteristics of cells, because there is an implicit requirement for an interaction between two inputs that yield a biological response that neither one alone can yield. As far as the candidate mechanism for the calcium mediated branch of the synergistic interaction, it is tempting to speculate that calcium-calmodulin dependent protein kinase (CaM Kinase) is involved, since its injection into type-B cells can induce the same biophysical alterations as learning, i.e., a calcium-dependent, long-term reduction in both I_A and $I_{K(Ca^{++})}$ (Acosta-Urquidi et al., 1983; Sakakibara et al., 1986). Furthermore, in hippocampal CA1 cells, CaM kinase is increased in activity 24 h after rabbit eye blink conditioning (Bank et al., in press).

Based on data we have accumulated from *Hermissenda* B cells and rabbit CA1 cells, we have developed a biochemical model of associative learning that incorporates the cardinal feature of associative learning: temporal contiguity between two stimuli (Fig. 5). In hippocampus, the CS pathway is hypothesized to act through the glutamanergic synapse because the glutamanergic synapse is enhanced after conditioning (LoTurco et al., 1988) and is the major neurotransmitter system in the hippocampus. In *Hermissenda*, the CS is light that is transduced directly by the photoreceptor, and it is the response to light that is altered as a result of conditioning. Both these CS pathways are presumed to be coupled to a G protein that causes the activation of phospholipase C, which in turn breaks down P1P2 into the two intracellular second messengers, IP3 and DG (Berridge et al., 1984). DG causes translocation of PKC and IP3 causes increases in intracellular calcium. Therefore, the CS causes a transient association of PKC with the membrane. If, however, a US occurs in conjunction with this event, stimulus (shock or rotation) will cause a simultaneous depolarization of the cell, which results in a calcium influx. The calcium influx will activate CaM Kinase, which causes a stable attachment of PKC to the membrane. This is presumed to occur via a calmodulin-dependent phosphorylation of PKC or its membrane anchoring proteins (Wolf and Sahyoun, 1986) so that PKC is no longer susceptible to the processes that normally detach it from the membrane, such as proteolysis (Melloni et al., 1985) or autophosphorylation (May et al., 1985). As previously stated, if the CS occurs alone, only a transient association of PKC with the membrane will result. If the US occurs alone, CaM kinase will only be transiently activated and returned to basal levels when intracellular calcium is buffered. The long-term association of PKC with the

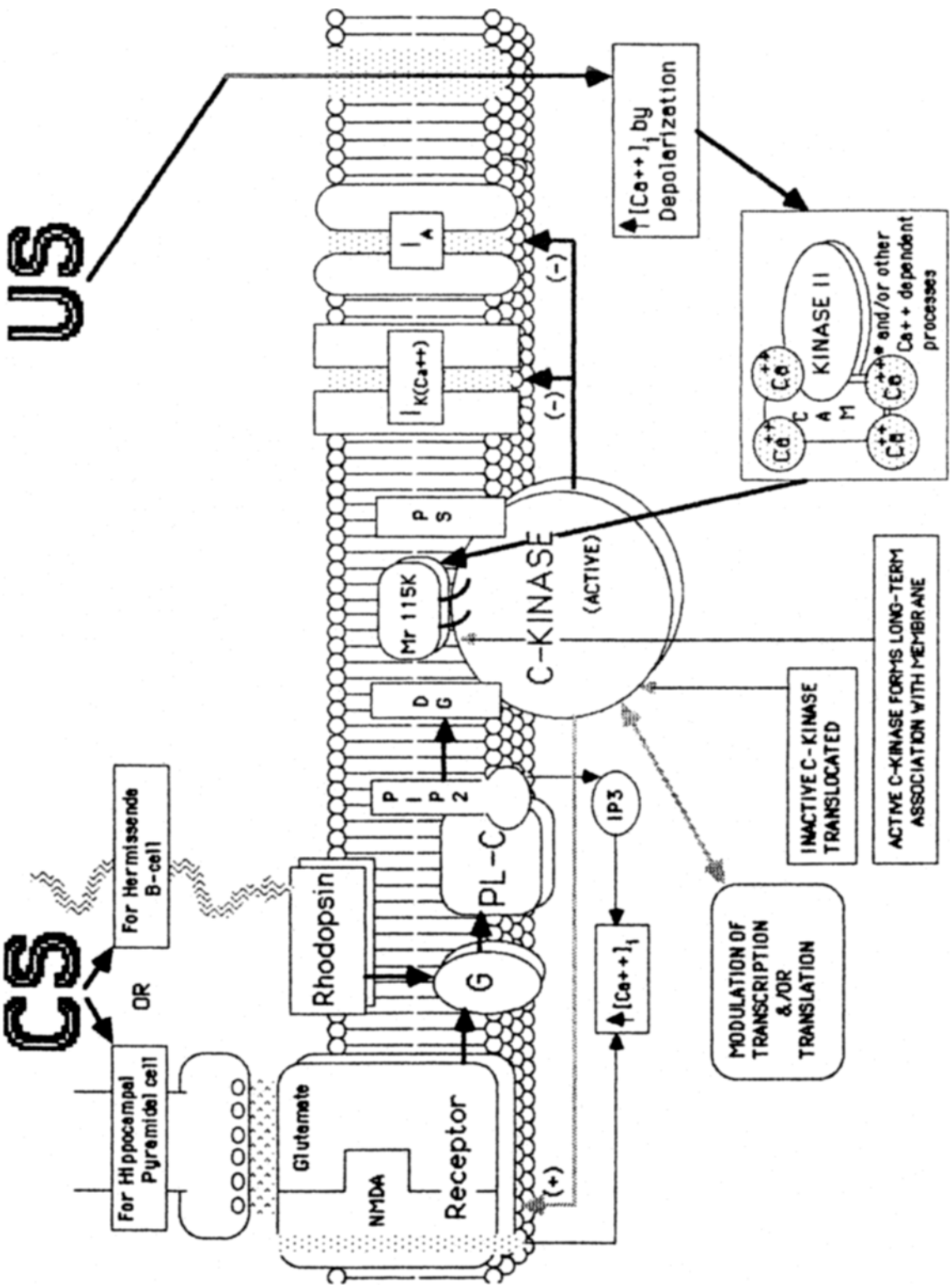

Fig. 5. Biochemical model of associative learning. The putative CS-activated pathway acts through the glutamanergic synapse in the case of the CA1 cells, and through light activation of rhodopsin in the B photoreceptor to activate diacylglycerol (DG). DG causes translocation of PKC from cytosol to membrane and IP_3 increases intracellular calcium concentrations. The CS-activated pathway causes a transient association of PKC with the membrane. The putative US-activated pathway, an increase in internal calcium concentration in response to depolarization, involves the activation of some calcium-dependent pathway (e.g., calmodulin-dependent kinase II). Concurrence of the CS-activated and US-activated pathways leads to a stable attachment of PKC via calcium-dependent phosphorylation of PKC or its membrane anchoring proteins (M_r 115 kD).

membrane can phosphorylate ion channels or their constituents, particularly in response to transient Ca^{++} elevation to increase cellular excitability by decreasing K^+ conductances or modulating synaptic conductances.

Protein kinaese C is also known to regulate the expression of gene products. For example, phorbol esters induce cellular growth, proliferation, and more specifically affect protein (Alkon et al., 1987) and DNA synthesis (*see* Nishizuka, 1984). This property of PKC could serve to bridge the temporal domain between immediate and intermediate effects of conditioning (i.e., hours to days) and the longest term effects, which last the lifetime of the organism. Specifically, long-term translocation to the plasma membrane (hours to days) could underly the immediate to intermediate effects of conditioning, whereas PKC translocation to the nuclear membrane (Thomas et al., 1986; Wood et al., 1986) could trigger the genome to cause permanent neural alterations that underly memories lasting from months to years. In support of this hypothesis, we have recently found that conditioning in *Hermissenda* results in an increase in mRNA synthesis 24 h after conditioning, which correlates highly with the degree of learning observed (Nelson and Alkon, 1987). Furthermore, *Hermissenda* conditioning has now been found to alter the synthesis of specific proteins, one of which is a substrate for PKC as well as CaM kinase (Nelson and Alkon, 1988). This protein (21,000 MW) has also been found to undergo a change of phosphorylation as a result of conditioning (Neary et al., 1981). PKC activation may be the trigger for this conditioning-specific increase in mRNA synthesis.

At the cellular level, the ordering of CS and US (i.e., activation of DG and calcium influx) required to cause long-term PKC translocation is not presently known. Perhaps either order will suffice to cause long-term translocation. The requirement for forward pairing of CS and US for behavioral conditioning may be subsumed by neural pathways and not biochemical pathways. However, in the case of associative LTP induced in hippocampal CA1 cells, neurotransmitter activation (DG formation) cannot follow depolarization (Ca^{++} influx) (Gustaffson et al., 1987).

Summary

PKC activation has been shown to mimic the biophysical consequences of classical conditioning in both rabbit hippocampus and *Hermissenda* type B cells. Furthermore, conditioning in rabbits results in the 24 h translocation of PKC from cytosol to membrane, which is probably responsible for mediating the biophysical consequences of conditioning. A model has been presented that suggests that long-term translocation of PKC occurs via the synergistic activation of a DG dependent pathway that activates PKC and a calcium dependent pathway that activates CaM kinase. Translocation of PKC to the plasma membrane, by altering ion channel properties, could subserve memory lasting for days, whereas translocation to the nuclear membrane could induce cellular change, by genomic regulation, lasting beyond days. We are, therefore, suggesting that protein kinase C may play a critical role in the formation of short, intermediate, and long-term associative memory.

References

Acosta-Urquidi J., Alkon D. L., and Neary J. T. (1984) Intrasomatic injection of a calcium calmodulin dependent protein kinase stimulates biophysical effects of associative learning in a *Hermissenda* photoreceptor. *Science* **224**, 1254–1257.

Akers R. F., Lovinger D. M., Colley P. A., Linden D. J., and Routtenberg A. (1986) *Science* **231**, 587–589.

Alkon D. L. (1974) Associative training in *Hermissenda*. *J. Gen. Physiol.* **64**, 70–84.

Alkon D. L. (1984) Calcium mediated reduction of ionic currents: A biophysical memory trace. *Science* **226**, 1037–1045.

Alkon D. L., Sakakibara M., Forman R., Harrigan J., Lederhendler I., and Farley J. (1985) Reduction of two voltage-dependent K^+ currents mediates retention of a learned association. *Behav. Neural Biol.* **44**, 278–300.

Alkon D. L., Kubota M., Neary J. T., Naito S., Coulter D., and Rasmussen H. (1986) C-Kinase activation prolongs CA^{++}-dependent inactivation of K^+ currents. *Proc. Natl. Acad. Sci. USA* **84**, 6947–6951.

Alkon D. L., Naito S., Kubota M., Chen C., Bank B., Smallwood J., Gallant P., and Rasmussen H. (1988) Regulation of *Hermissenda* K^+ channels by cytoplasmic and membrane-associated protein kinase C. *J. Neurochem.* **51**, 903–917.

Bank B., DeWeer A., Kuzirian A. M., Rasmussen H., and Alkon D. L. (1988) Classical conditioning induces translocation of protein kinase C in rabbit hippocampal CA1 cells. *Proc. Natl. Acad. Sci. USA* **85**, 1988–1992.

Baraban J. M., Snyder S. H., and Alger B. E. (1985) Protein kinase C regulates ionic conductances in hippocampal pyramidal neurons electrophysiological effects of phorbol esters. *Proc. Natl. Acad. Sci. USA* **82**, 2538–2342.

Berger T. W. (1984) Long-term potentiation of hippocampal synaptic transmission accelerates behavioral learning. *Science* **224**, 627–630.

Berger T. W. and Orr B. W. (1983) Hippocampectomy selectively disrupts membrane response. *Behav. Brain Res.* **8**, 49–68.

Berger T. W., Rinaldi P. C., Weisz D. J., and Thompson R. F. (1983) Single unit analysis of different hippocampal cell types during classical conditioning of rabbit nictitating membrane response. *J. Neurophys.* **50**, 1197–1219.

Berridge M. J. (1984) Inositol triphosphate and diacylglycerol as second messengers. *Biochem. J.* **220**, 345–360.

Coulter D. A., LoTurco J. J., Kubota M., Disterhoft J. F., Moore J. W., and Alkon D. L. (1987) Reduction of the AHP in CA1 pyramidal cells is induced by classical conditioning. *J. Neurophys.*

Coulter D. A., Disterhoft J. F., and Alkon D. L. (1986) Decreased calcium entry and potassium conductance contribute to AHP reduction with conditioning in rabbit. *Soc. Neurosc. Abstr.* **12**, 181.

Crow T. J. and Alkon D. L. (1978) Retention of an associative behavioral change in *Hermissenda*. *Science* **201**, 1239–1241.

Disterhoft J. F., Coulter D. A., and Alkon D. L. (1986) Conditioning-specific membrane changes of rabbit hippocampal neurons measured in vitro. *Proc. Natl. Acad. Sci. USA* **83**, 2733–2737.

Farley J., Richards W. G., Ling L., Liman E., and Alkon D. L. (1983) Membrane changes in a single photoreceptor during acquisition cause associative learning in *Hermissenda*. *Science* **221**, 1201–1203.

Gormezano I., Schneiderman N., Deaux E., and Fuentes I. (1962) Nictitating membrane: classical conditioning and extinction in the albino rabbit. *Science* **138**, 33–34.

Gormezano I., Prokasy W. F., and Thompson R. F. (1986) Classical conditioning III. Behavioral, neurophysiological, and neurochemical studies in the rabbit. Erlbaum, Hillsdale, NJ.

Gustaffson B., Wigstrom H., Abraham W. C., and Huang Y. Y. (1987) Long-term potentiation in the hippocampus using depolarizing current pulses as the conditioning stimulus to single volley synaptic potentials. *J. Neurosc.* **7(3)**, 774–780.

Hotson J. R. and Prince D. A. (1980) A calcium activated hyperpolarization follows repetitive firing in hippocampal neurons. *J. Neurophys.* **43**, 409–419.

Kaibuchi K., Takai Y., Sawamura M., Hoshijima M., Fujikura T., and Nishizuka Y. (1983) Synergistic functions of protein phosphorylation and calcium mobilization in platelet aggregation. *J. Biol. Chem.* **258**, 6701–6704.

Kojima I., Kojima K., Kreutter D., and Rasmussen H. (1984) The temporal integration of the aldosterone secretory response to angiotensin occurs via two intracellular pathways. *J. Biol. Chem.* **259**, 14448–14457.

Kojima I., Kojima K., and Rasmussen H. (1985) Role of calcium fluxes in the sustained phase of angiotensin II mediated aldosterone secretion from adrenal glomerulose cells. *J. Biol. Chem.* **260**, 9177–9184.

Lancaster B. and Adams P. R. (1986) Single electrode voltage-clamp of slow AHP current in rat hippocampal cells. *J. Neurophys.* **55**, 1268.

Lederhendler I., Gart S., and Alkon D. L. (1986) Classical conditioning of *Hermissenda* origin of a new response. *J. Neurosci.* **6**, 1325–1331.

LoTurco J. J., Coulter D. A., and Alkon D. L. (1988) Enhancement of synaptic potentials in rabbit CA1

pyramidal neurons following classical conditioning. *Proc. Natl. Acad. Sci. USA* **85**, 1672–1676.

Lovinger D. M., Akers R. F., Nelson R. B., Barnes C. A., McNaughton B. L., and Routtenberg A. (1985) A selective increase in phosphorylation of protein F1, a kinase C substrate, directly related to three day growth of long-term synaptic enhancement. *Brain Res.* **343**, 137–143.

Malenka R. C., Madison D. V., Andrade R., and Nicoll R. A. (1986) Phorbol esters mimic some cholinergic actions in hippocampal pyramidal neurons. *J. Neurosci.* **6**, 475–480.

Malenka R. L., Madison D. V., and Nicoll R. A. (1986) Potentiation of synaptic transmission in the hippocampus by phorbol esters. *Nature* **321**, 175–177.

Mamounas L. A., Thompson R. F., Lynch G., and Baudry M. (1984) Classical conditioning of the rabbit's eyelid response increase glutamate receptor binding in hippocampal receptor membranes. *Proc. Natl. Acad. Sci. USA* **81**, 2548–2552.

McCormick D. A. and Thompson R. F. (1984) Cerebellum: essential involvement in the classically conditioned eyelid response. *Science* **223**, 296–299.

Melloni E., Pontremoli S., Michetti M., Sacco O., Sparatore B., Salamino F., and Horecker B. L. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 6435–6439.

Naito S., Bank B., and Alkon D. L. (1988) Transient and persistent depolarization-induced changes of protein phosphorylation in a molluscan nervous system. *J. Neurochem.* **50**, 704–711.

Neary J. T., Crow T. J., and Alkon D. L. (1981) Change in a specific phospoprotein band following associative learning in *Hermissenda*. *Nature* **293**, 658–660.

Neary J. T., Naito S., Deweer A., and Alkon D. L. (1986) Calcium/diacylglycerol activated phospholipid dependent protein kinase in the *Hermissenda* CNS. *J. Neurochem.* **47**, 1405–1411.

Nelson T. and Alkon D. L. (1988) Learning dependent changes in mRNA turnover in *Hermissenda* photoreceptors. *PNAS*.

Nelson T. and Alkon D. L. (1988) Protein and mRNA changes by associative conditioning of *Hermissenda*. *Soc. Neurosci. Abstracts* 910.

Orr W. B. and Berger T. W. (1985) Hippocampectomy disrupts the topography of conditioned nictitating membrane responses during reversal learning. *Behav. Neurosci.* **99**, 35–45.

Port R. L., Mikhail A. A., and Patterson M. M. (1985) Differential effects of hippocampectomy on classically conditioned rabbit nictitating membrane response related to interstimulus interval. *Behav. Neurosci.* **99**, 200–208.

Port R. L., Romano A. G., Steinmetz J. E., Mikhail A. A., and Patterson M. M. (1986) Retention and acquisition of classical trace conditioned responses by rabbits with hippocampal lesions. *Behav. Neurosci.* **100**, 745–752.

Port R. L. and Patterson M. M. (1984) Fimbrial lesions and sensory preconditioning. *Behav. Neurosci.* **98**, 584–589.

Richards W., Farley J., and Alkon D. L. (1984) Extinction of associative learning in *Hermissenda*: Behavior and Neural correlates. *Behav. Brain Res.*

Routtenberg A., Lovinger D., and Steward O. (1985) Selective increase in phosporylation of a 47 kD protein (F1) directly related to long-term potentiation. *Behav. Neural Biol.* **43**, 3–11.

Sakakibara M., Alkon D. L., Delorenzo R., Goldenring J. R., Neary J. T., and Heldman E. (1986) Modulation of calcium-mediated inactivation of ionic currents by Ca^{++}/calmodulin-dependent protein kinase II. *Biophy. J.* **50**, 319–327.

Schmaltz L. W. and Theios J. (1972) Acquisition and extinction of a classically conditioned response in hippocampectomized rabbits. *J. Comp. Physiol. Psych.* **79**, 328–333.

Soloman P. R., Vander Schaff E. R., Nobre, Thompson R. F., and Weisz D. J. (1986) Hippocampus and trace conditioning of the rabbit's classically conditioned nictitating membrane response. *Behav. Neurosci.* **100**, 729–744.

Soloman P. R., Soloman S. D., Vander Schaff E. R., and Perry H. E. (1983) Altered activity in the hippocampus is more detrimental to classical conditioning than removing the structure. *Science* **200**, 329–331.

Soloman P. R. (1977) Role of the hippocampus in blocking and conditioned inhibition of the rabbit's nictitating membrane response. *J. Comp. Physiol. Psych.* **91**, 407–417.

Soloman P. R. and Moore J. W. (1975) Latent inhibition and stimulus generalization of the classically conditioned nictitating membrane response in rabbits following hippocampal ablation. *J. Comp. Physiol. Psychol.* **89**, 1192–1203.

Squire L. R. and Zola-Morgan S. (1982) The neurology of memory: The case for correspondence between the findings for human and non-human pri-

mates. *The Physiological Basis of Memory* (Deutsch J. A., ed.), Academic, New York, pp. 199–268.

Thompson R. F., Clark G. A., Donnegan N. H., Lavond D. G., Madden J., Mamounas L. A., Mauk M. D., and McCormick D. A. (1984) Neuronal substrates of basic associative learning. *Neuropsychology of Memory* (Butters N. and Squire L., eds.), Guilford, New York, pp. 424–442.

Wolf M., Cuatrecasas P., and Sahyoun N. (1985) Interaction of protein kinase C with membranes is regulated by Ca^{++}, phorbol esters, and ATP. *J. Biol. Chem.* **260,** 15718–15722.

Wolf M. and Sahyoun N. (1986) Protein kinase C and phosphatidylserine bind to Mr 110,000/115,000 polypeptides enriched in cytoskeletal and postsynaptic density preparations. *J. Biol. Chem.* **261,** 13327–13332.

Wood J. G., Girard P. R., Mazzei G. J., and Kuo J. F. (1986) Immunocytochemical localization of protein kinase C in identified neuronal compartments of rat brain. *J. Neurosci.* **6,** 2571–2577.