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Protein kinase C: a nexus in the biochemical events that underlie associative learning

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Abstract. We have proposed that protein kinase C, an enzyme critical to cell regulation of growth, secretion and differentiation, is a part of a sequence of molecular events that underlie learning and memory. Electrophysiological, biochemical and neuro-imaging methods have been employed to show that the enzyme changes its distribution as a result of memory storage within the neural networks that are necessary for the acquisition and performance of various learning tasks in several species. We propose here, a model of protein kinase C as a molecular signal for the association of synaptic input that is parsimonious with the recent data, mainly from our laboratory, concerning its function in memory formation.

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INTRODUCTION

Protein kinase C (PKC) is a calcium-lipid dependent enzyme first characterized by Nizhisuka (Takei et al. 1979) in 1979. It has since been found to play an important role in regulating cellular function in most eukaryotic systems (Nishizuka 1988, Alkon and Rasmussen 1988, Nishizuka 1992) and is characterized by requiring both an appropriate lipid milieu and a Ca^{2+} signal for physiological activation (Hannun et al. 1985, Morimoto et al. 1988). Recently, PKC has been found by us to play an important role in associative learning (Olds et al. 1989, Olds et al. 1990, McPhie et al. in press, Sharenberg et al. 1991) as assessed by a variety of imaging techniques. Other methodologies (Nelson et al. 1987, Bank et al. 1988, Wehner et al. 1990a, Wehner et al. 1990b) have tended to confirm the evolving hypothesis that PKC activation may serve as a critical step in the chain of biological events that leads to memory formation.

We propose here a model in which PKC serves as a molecular signal for concurrent presynaptic input, most likely in concert with other protein regulators. Thus, PKC activation can act as an index for two different sensory stimuli that are associated in the time domain in a manner similar to that suggested by Friedrich and co-workers (1987, 1990). Such a function could have important theoretical implications for the development of artificial neural networks (Alkon et al. 1990). The proposed model postulates that PKC, via tertiary messenger cellular pathways, eventually lead to extremely long-term learning-specific changes in neuronal gene expression and morphology.

THE ENZYME: A SERINE-THREONINE PROTEIN KINASE

Molecular profile

Depending upon the species from which PKC is isolated, this enzyme has a molecular weight of between 80 and 90-kD (Nishizuka 1988). The gene is part of a large gene family of which at least 10 different isoforms have been isolated (for review see Nishizuka 1992). There is a close correspondence between the different genes for PKC that have been isolated and the different isozymes of the protein (Huang F.L.et al. 1987, Woodgett and Hunter 1987, Hidaka et al. 1988, Huang F.L. et al. 1988, Nakabayashi and Huang K.P. 1988, Nishizuka 1988, Stichel and Singer 1988, Yoshida et al. 1988, Huang F.L. et al. 1989, Huang K.P. et al. 1989, Yoshida et al. 1988, Roth et al. 1989, Saito et al. 1989). At least one of the isozymes (corresponding to the γ isoform of the gene) is specific to brain and is highly enriched in the mammalian hippocampus and neocortex (Nishizuka 1988, Stichel and Singer 1988, Roth et al. 1989b, Nishizuka 1988). Several of the other isozymes are also highly prevalent in brain tissue and, on the basis of hybridization studies, appear to exhibit distinctive neuroanatomical distributions (Stichel and Singer 1988, Craig et al. submitted).

Activation *via* membrane association

Because PKC belongs to a class of proteins that are amphitrophic (i.e. composed of regions which are both hydrophobic and hydrophillic; Brumfeld and Lester 1990, Lester et al. 1990), the localization of the enzyme to the membrane milieu has been thought to be reflective of the enzyme's state of activation. Early studies clearly demonstrated that the activity of the enzyme dependended upon the phospholipids phosphotidylserine (PS) and phosphotidyl choline (PC; Hannun et al. 1985), both of which occur in the plasma membrane (Hannun et al. 1985). In addition, the enzyme shows a marked increase in its phosphorylating activity upon addition of diacylglycerol (DG) to the lipid surrounding the membrane-associated (i.e. translocated) form the enzyme (Hannun et al. 1985). For maximal activity however, some isozymes of PKC require Ca^{2+} (approximately 5 μM) in the microenvironment of the enzyme (Hannun et al. 1985). The phorbol esters and cis-fatty acids also pharmacologically activate

PKC (Morimoto et al. 1988). This activation occurs *via* high affinity binding near the DG binding site in the C1 or zinc-finger portion of the molecule (Huang K.P.1989).

CELLULAR SIGNAL TRANSDUCTION: PKC AT THE JUNCTURE BETWEEN THE NEURON AND ITS IMMEDIATE ENVIRONMENT

The active phorbol ester compounds were initially characterized as tumor promoters (Hecker, 1978, Castagna et al. 1982) and after the initial identification of PKC as the phorbol ester receptor (Ashendel et al. 1983) it became clear that the enzyme itself played a major role in the cellular transformation that underlies oncogenesis (for a review, see Nishizuka 1988). Subsequently it became evident that PKC is also important in cellular growth, secretion, and adhesion (see review Nishizuka 1988). These roles reflect the more general role of PKC in signal transduction. Extrinsic signals, such as phorbol esters or endogenous growth factors, are able to effect a kind of cellular memory (i.e. very long term changes in the cell) by the PKC activation process. These extrinsic signals are also highly regulated by cross-talk between PKC and other second and tertiary messenger systems within the cell (Nishizuka 1988).

Putative protein substrates for protein kinase C

Several important substrates for PKC have been discovered in vertebrate and invertebrate brain. These include PKC itself, which autophosphorylates on threonine residues (Schwartz and Greenberg 1987), and an 87- kD protein, MARKS, which has been characterized and is found to be heavily myristoylated, (Ouimet et al. 1990). The 87-kD protein is found throughout the brain, but is enriched in the piriform and entorhinal cortices, the amygdala, intralaminar thalamic nucleii, the hypothalamus and many aminergic nucleii. Ultrastructural analysis has shown this protein to be localized in axons and small dendrites endings, but not in somata and large dendrites (Ouimet et al. 1990). Recent evidence suggest that the phosphorylation of this protein substrate for PKC may be specifically decreased in Alzheimer's Disease (Cole et al. 1988).

Another major substrate for PKC is GAP43 which is specifically phosphorylated by PKC at serine 41 (Coggins and Zwiers 1989). This substrate protein occurs in presynaptic nerve endings in the CNS. It is especially enriched in the mammalian hippocampus (Routtenberg 1986), and its phosphorylation has been shown to parallel long term potentiation (LTP, Lovinger et al. 1986, Lovinger and Routtenberg 1988). 1988). It has also been observed that GAP43 forms gradients such that its phosphorylation state increases along the cortical visual processing pathways of monkeys (Nelson et al. 1987) and in growth cones of regenerating axons, where it is also a substrate for PKC (Van Lookeren et al. 1989).

Finally, an intriguing PKC substrate of 20kD has been described both in canine cerebral cortex (Suzuki and Siekevitz 1989), in the rabbit hippocampus, and in the nudibranch *Hermissenda crassicornis* (Neary et al. 1981, Nelson et al. 1990, Nelson et al. in preparation). In the canine, this substrate has been shown to be densely localized in postsynaptic densities. In Hermissenda, this substrate shows learning-specific increases in its degree of phosphorylation, reduces K^+ conductances in a manner that mimics Pavlovian conditioning and manifests a GTP-ase activity (Nelson et al. 1990). The potential identity between the canine, rabbit and molluscan substrates still remains to be established.

Neuroanatomical localization

The high affinity of the active phorbol esters for PKC has also led to mapping studies in which [^{3}H]-phorbol-12,13-dibutyrate ([^{3}H]PDBU) has been used as a radioligand (Worley et al. 1986a,

El-Fakahany et al. 1988, Huang K.P. et al. 1988, Olds et al. 1989, Onodera et al. 1989, Olds et al. 1990). The results of these autoradiographic mapping studies have been largely confirmed by less quantitative immunohistochemical studies with the use of antibodies to specific isozymes of PKC. In general, PKC in the mammalian nervous system is highly enriched in the superficial layers of the neocortex, the hippocampus and the purkinje cells of the cerebellum (Huang F.L. et al. 1987, Huang F.L. et al. 1988, Huang K.P. et al. 1988, Saito et al. 1988, Stichel and Singer 1988, Huang F.L. et al. 1989, Huang K.P. 1989, Roth et al. 1989a,b, Saito et al. 1989). Immunohistochemical studies at the ultrastructural level have demonstrated that PKC is associated with the plasma membrane in axons and dendrites and at the somata (Yoshida et al. 1988). Some studies have shown at least one isozyme is associated with the Golgi apparatus (Saito et al. 1989).

PKC AND LEARNING

Learning defined

Learning can be defined operationally as a relatively permanent change in an animal's behavior as a result of experience. At its most basic level, learning consists of the formation of associations between stimuli that occur together or at least near one another in time. The associative nature of learning was described early on by Pavlov (Pavlov 1927) and later by Hebb (Hebb 1949). Over the years classical (associative) conditioning has been used by many investigators to assess learning and its correlates in a wide variety of animals ranging from the marine snail to mammals (Olds 1972, Olds et al. 1972, Olds 1975, Alkon 1980, Alkon et al. 1982, Takenda and Alkon 1982, Crow 1983, Crow and Offenbach 1983, Crow 1985, Farley and Auerbach 1986, Collin et al. 1988, Matzel et al. 1989).

Invertebrate injection studies

Experiments on the marine nudibranch, *Hermissenda crassicornis* were the first to demonstrate that PKC might play an important role in the neuronal changes that subserve associative learning. *Hermissenda* can be classically conditioned to associate light with rotation leading to the development of a new conditioned response (shortening of the animal's foot). The biological basis for this conditioned response has been demonstrated to reside within the B cell, which lies at the convergence point of information flow between the visual and vestibular sensory pathways (for a review see Alkon 1989). Microinjection of PKC directly into the B photoreceptor, concurrent with the delivery of a Ca^{2+} load (via light flash) mimicked the effects of Pavlovian conditioning upon these cells (Alkon et al. 1988). Specifically, the Ca^{2+}-dependent K^{+} ionic current showed a profound decrease in conductance, as had been previously shown in animals that had received repeated paired light and rotation stimuli, but not in control animals (Alkon 1984, Alkon et al. 1985).

The involvement of PKC in Hermissenda classical conditioning was recently been confirmed autoradiographically. In this case, Pavlovian conditioning also produced an increase in [^{3}H]PDBU binding as assessed by computerized silver grain image analysis within these same B photorecptors that had previously been shown to undergo biophysical changes with learning (McPhie et al. in press). This change was behaviorally specific, in that control group animals did not exhibit the change, and also anatomically specific at the cellular level since only cells previously demonstrated to be crucial to the development of the conditioned response showed the change.

PKC activity assays and learning

In parallel with the results from the invertebrate studies, a steady-state increase in PKC activity was found to be associated with the membrane in the CA1 hippocampal cell field of rabbits that had received 3 days of Pavlovian conditioning of the nictitating membrane (Bank et al. 1988). This sustained increase lasted as long as 24 h after the last training trial. These results taken together with the

demonstration that a steady state decrease in I_{AHP} in CA1 pyramidal cells occurs in similarly conditioned rabbits, but not in control animals (Disterhoft et al. 1986, Loturco et al. 1988, Coulter et al. 1989, Sanchez-Andres and Alkon 1992) and that PKC-activating phorbol esters can mimic the effect of Pavlovian conditioning on the I_{AHP} (Alkon et al. 1986), suggested an important role for PKC in associative memory storage within the hippocampus. Furthermore, voltage clamp studies have indicated that the I_{AHP} is subserved by a K^+ current (Sanchez-Andres and Alkon 1992). Thus, the ionic current modified by associative conditioning is similar in both *Hermissenda* and the rabbit.

Quantitative autoradiographic studies

The advent of sophisticated workstation-based image analysis and the concomittant development of high affinity radioligands for PKC (Worley et al. 1986a,b), has made it possible to actually map the distributional changes in this enzyme after associative conditioning. In our laboratory, we have employed [^{3}H]PDBU quantitative autoradiography to study activated PKC in rabbits that had received 3 days of Pavlovian conditioning trials. Image analysis revealed a dramatic increase in activated PKC in the CA1 region of the hippocampus from conditioned animals but not control animals. This change in the distribution of the enzyme within the hippocampus was both long-lasting and dynamic. While the increase was primarily localized in the area of the CA1 pyramidal cell somata 24 h after conditioning, the area of increased binding shifted to the basilar dendrites 72 h after conditioning (Olds et al. 1989).

In additional studies, the same methodology was used to study the initial acquisition of the conditioned response in rabbits. In contrast to the CA1 specific increase in membrane associated PKC seen 24 h and 72 h after 3 days of classical conditioning, rabbits studied after just 80 conditioning trials showed an increase in PKC membrane association specific to the stratum oriens of CA3 but not CA1 (Scharenberg et al. 1991).

A hippocampal-specific change in PKC membrane association was also seen in rats that had received discrimination training in water maze procedure, but not in control animals (Olds et al. 1990). In this case, both spatial and cued discrimination procedures produced a significant decrease in [^{3}H]PDBU binding in the CA3 cell field. Both tasks required an intact hippocampus. What was extraordinary about this finding in relation to the previous result in rabbit hippocampus was the involvement of hippocampal PKC in a different species that was performing a completely different task. This suggests that the enzyme's role in memory storage is not simply an artifact of the learning task but reflects a more generalized involvement of PKC in the underlying biological mechanism. This finding has recently been supported by work with inbred mouse mutants (Wehner et al. 1990a, Wehner et al. 1990b) in which Mice with superior spatial-learning ability (as assessed by a water maze task) were shown to have significantly increased hippocampal PKC activity.

PKC and Alzheimers disease

Alzheimer's Disease (AD) is characterized by a decline in cognitive functions (especially memory) in combination with neuronal cell death in the basal forebrain, neocortex, and hippocampus over a relatively long period of time (Katzman 1986). Recent analysis of human tissue obtained post mortem from AD patients and age-matched controls has revealed a striking decrease in PKC as measured by radioactive phorbol ester binding (Cole et al. 1988) as well as by quantitative immunohistochemistry (Masliah et al. 1990). This decrease does not simply reflect neuronal cell death since fibroblasts derived from AD patients also have significantly reduced PKC compared to those from control patients (Huynh et al. 1989). What is most intriguing about the most recent clinical results is that the decrease seems to be specific to the ßII isozyme of PKC in tissue from AD human hippocampus and cortex when compared to controls (Masliah et al. 1990). Thus, the amount of a specific isozyme of PKC has

been demonstrated to be significantly decreased in a human disorder that not only involves memory, but also a specific neuropathology in the hippocampus.

Long term potentiation

In addition to its potential role in associative learning, PKC has also been shown to be involved in a laboratory model of synaptic plasticity known as long term potentiation (LTP). LTP refers to the long term enhancement of the excitatory postsynpatic potential produced by rapid tetanic stimulation of presynaptic axons both within the hippocampus and more recently in other neuroanatomic structures (Bliss and Garner-Medwin 1973, Levy and Steward 1979). Routtenberg and his colleagues observed increases in the phosphorylation of the PKC substrate protein GAP43 that parallels the development of LTP (Routtenberg 1986, Lovinger and Routtenberg 1988, Linden and Routtenberg 1989). Furthermore, application of phorbol esters prolonged the maintenance of LTP (Malenka et al.1986, Routtenberg et al 1986, Malenka et al. 1987). It was even suggested that the effects of phorbol esters mimicked entirely the synaptic enhancement of LTP (Malenka et al. 1986) since in initial studies, the application of phorbol ester to hippocampal slices resulted in a saturation of synaptic enhancement such that further tetanic stimulation would elicit no further LTP.

Supporting the theory that PKC is involved at least in the maintenance of LTP, pharmacological blockers of PKC such as H7, polymyxin B, sphingosine and melitin all inhibit the maintenance of LTP after its initial PKC independent initiation (Malenka et al. 1986, Lovinger and Routtenberg 1988). However, all of these inhibitors may have nonspecific effects on other kinases as well as PKC.

A MODEL FOR PKC ACTIVATION IN ASSOCIATIVE LEARNING

The above discussion inevitably leads to the question of how PKC fits in to the chain of molecular events in neural elements that result in memory storage. We haved proposed a multifunctional model that is described below (Alkon 1989, Olds et al. 1989).

Stage I: Dendritic activation of PKC

In the first stage of the proposed model for memory storage, signals from the immediate environment are filtered for salience on an unconscious level. This filtering process takes into account physiological variables such as the current drive state of the animal. Thus, a tone is perceived to be important, or salient, to a rabbit only if it occurs within the appropriate environmental or behavioral milieu. The neurobiological substrate for such filtering of stimuli might involve either the cholinergic or noradrenergic systems of the mammalian nervous system since both of them project extensively not only onto the cells of the hippocampus, but also onto the entire neocortex (Lehman et al. 1984). Furthermore, both the cholinergic and noradrenergic systems can affect PKC (Hashimoto et al. 1988). Signals that pass through this filtering process eventually converge on the dendritic branches of certain neurons that, for heuristic purposes, we call neural storage elements (NSE's). Signals that occur together or nearly together in time activate multiple excitatory amino acid receptors (including quisqualate and NMDA) which together (via phospholipase C, D and A_2) provide the necessary activational conditions for PKC. Hypothetically, this activated, or membrane-associated form of PKC becomes localized in the area (or patch) of the NSE membrane that received the two signal inputs (Fig.1left) and no longer depends on other cofactors for further activation (Nelsestuen and Bazzi 1989). In other words, once PKC has become associated with the membrane (due to complexing with its cofactors) it is constitutively activated. This initial Stage I activation corresponds to the acquisition data of Scharenberg et al (1990) in CA3 stratum oriens. Other synaptic input onto the NSE (not carrying information about salient sensory stimuli) may play a critical role in gating this PKC activation.

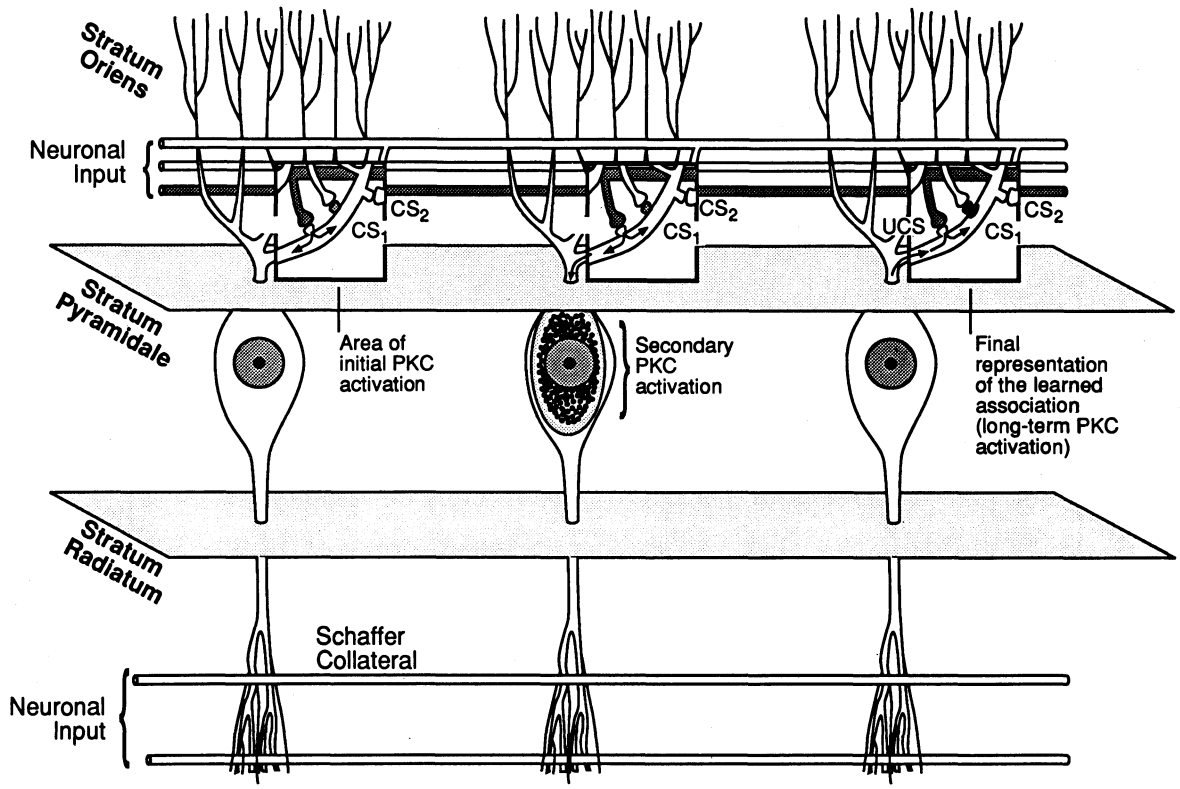

Fig. 1. Schematic depiction of pyramidal cells in the CA1 region of the hippocampus illustrating a sequential activation of PKC within different intracellular spatial domains. Left, initial cotemporal input of both CS1 and UCS results in local activation of PKC at the postsynaptic NSE. Middle, after the initial stimulation of PKC at the NSE, a sequence of cellular events causes alterations in cellular transport mechanisms and additionally results in an apparent increase in membrane-associated PKC associated with cell body organelles. Right, finally, in the last stage of "consolidation," specifically targeted, newly synthesized proteins (possibly including PKC) make their way back to dendritic region of the initially activated NSE, resulting in a long-term change in the biophysical characteristics of the NSE in response to new inputs via the CS1 input pathway. CS2 represents input from a temporally unpaired stimulus.

The association and localization of PKC with the NSE described above leads to PKC-mediated phosphorylation of Ca^{2+}- dependent K^+ channels (either directly) or possibly of intermediate proteins, such as the recently discovered cp20 (Nelson et al. 1990), which then cause K^+ channel inactivation. This stage renders the patch of NSE more electrically responsive to both of the two initial input signals (either together or alone) and, in effect, indexes an association between them. Thus, in Stage I, after stimulus pairing, the patch of NSE generates a postsynaptic potential to both of the original synaptic inputs that is significantly greater than the synaptic potential generated by either input alone before conditioning. Since the NSE has been potentiated, one of the two stimuli will now substitute of the previous effect of both stimuli at the same time.

Stage II: PKC activation of retro-messages

In Stage II, consolidation (Fig. 1middle), the well-described process of PKC downregulation (Nishizuka 1988) begins to occur in the patch of activated NSE. This may be due to proteolytic degradation of the activated PKC and to the dephosphorylation of either the K^+ channels or the intermediate target proteins by phosphatases. However, the same PKC down-regulation process that results,essentially in the homeostatic reversal of Stage I, causes an alteration in neuronal transport

mechanisms. This alteration in neuronal transport mechanisms within the cell somata may be caused by either a molecular or electrical retro-message such that specific molecules (possibly including new PKC molecules and/or cp20) are targeted and then subsequently transported to the NSE (Fig. 1right). This stage of memory storage corresponds to the 72 h data on hippocampal PKC distribution in classically conditioned rabbits where [^{3}H]PDBU binding shifted from the area of the CA1 pyramidal somata (stratum pyramidale) to the area of the CA1 pyramidal dendrites(stratum oriens; Olds et al. 1989). This stage is also supported by recent findings in our laboratory that injection of cp20 into invertebrate neurons profoundly affects axonal transport (Alkon personal communication) and by immunohistochemical studies showing the ß PKC isozyme to be associated with the Golgi apparatus (Saito et al. 1989). This de novo insertion of new proteins into the original NSE renders the original biophysical change that occurred in Stage I more permanent.

Stage III: Genomic alterations

In the final stage of memory storage, oncogene activation occurs leading to the transcritional up-regulation of specific genes. In support of this hypothesis, PKC activation has been shown to activate FOS/JUN binding to the AP1 promoter site on some genes (Auwerx et al. 1990). The protein products of such genomic regulation could be transported in a similar manner as in Stage II to the NSE, resulting in much longer term changes.

CONCLUSIONS

Evidence from a variety of different preparations and behavioral paradigms now seems to leave little doubt that PKC is somehow involved in the sequence of events that occur within neurons during memory formation. Here, we have reviewed some of this evidence. The changes in PKC distribution that seem to parallel associative learning, occur in nerve cells which have been independently demonstrated to be at critical convergence points in the association process (i.e. at the juncture of the conditioned stimulus and unconditioned stimulus pathways in the parlance of Pavlovian conditioning). A variety of methodologies have been used to track the activity and distribution of PKC within these nerve cells, and taken together seem to suggest that PKC plays amajor role in the memory storage process.

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