Cellular/Molecular

Synergistic Control of Protein Kinase Cγ Activity by Ionotropic and Metabotropic Glutamate Receptor Inputs in Hippocampal Neurons

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Conventional protein kinase C (PKC) isoforms are abundant neuronal signaling proteins with important roles in regulating synaptic plasticity and other neuronal processes. Here, we investigate the role of ionotropic and metabotropic glutamate receptor (iGluR and mGluR, respectively) activation on the generation of Ca^{2+} and diacylglycerol (DAG) signals and the subsequent activation of the neuron-specific PKCγ isoform in hippocampal neurons. By combining Ca^{2+} imaging with total internal reflection microscopy analysis of specific biosensors, we show that elevation of both Ca^{2+} and DAG is necessary for sustained translocation and activation of EGFP (enhanced green fluorescent protein)-PKCγ. Both DAG production and PKCγ translocation were localized processes, typically observed within discrete microdomains along the dendritic branches. Markedly, intermediate-strength NMDA receptor (NMDAR) activation or moderate electrical stimulation generated Ca^{2+} but no DAG signals, whereas mGluR activation generated DAG but no Ca^{2+} signals. Both receptors were needed for PKCγ activation. This suggests that a coincidence detection process exists between iGluRs and mGluRs that relies on a molecular coincidence detection process based on the corequirement of Ca^{2+} and DAG for PKCγ activation. Nevertheless, the requirement for costimulation with mGluRs could be overcome for maximal NMDAR stimulation through a direct production of DAG via activation of the Ca^{2+}-sensitive PLCδ (phospholipase Cδ) isoform. In a second important exception, mGluRs were sufficient for PKCγ activation in neurons in which Ca^{2+} stores were loaded by previous electrical activity. Together, the dual activation requirement for PKCγ provides a plausible molecular interpretation for different synergistic contributions of mGluRs to long-term potentiation and other synaptic plasticity processes.

Key words: PKC; calcium; diacylglycerol; total internal reflection microscopy; glutamate; synaptic plasticity

Introduction

Pharmacological, electrophysiological, and genetic studies have identified protein kinase Cs (PKCs) as important regulators for neuronal plasticity processes such as long-term potentiation (LTP) and long-term depression (Hu et al., 1987; Malinow et al., 1989; Abeliovich et al., 1993; Son et al. 1996; Malenka and Nicoll, 1999; Soderling and Derkach, 2000; Ali and Salter, 2001; Hrabetova and Sacktor, 2001; MacDonald et al., 2001; Carroll and Zukin, 2002). This study focuses on PKCγ, a conventional PKC isoform that is selectively expressed in most neurons including hippocampal pyramidal neurons and cerebellar Purkinje cells (Abeliovich et al., 1993; Roisin and Barbin, 1997; Saito and Shirai, 2002) and likely mediates the phosphorylation of a broad range of effectors such as cell surface receptors, ion channels, transcription factors, as well as cytoskeletal proteins.

Interestingly, the dual regulation of conventional PKCγ by Ca^{2+} and diacylglycerol (DAG) (Nishizuka, 1988, 1995; Newton, 1995; Mellor and Parker, 1998; Oancea and Meyer, 1998) raises the possibility that PKCγ acts as a molecular coincidence detector that integrates signals from ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs) (i.e., the main regulators of these two second messengers in central synapses). The synergistic interplay between the two families of receptors is particularly evident in the presence of moderate synaptic activity, when LTP induction requires the pairing of iGluR and mGluR activation (Wilsch et al., 1998; Kotecha et al., 2003).

In this study, we combined molecular and optical approaches to investigate translocation of PKCγ to plasma membrane, where the kinase is active. In particular, we used the narrow excitation depth of the evanescent field generated by total internal reflection microscopy (TIRM) (Axelrod, 1981; Toomre and Manstein,

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2001) to monitor the translocation of proteins fused with the enhanced green fluorescent protein (EGFP), which served as readouts for second messenger production as well as PKCγ activation in soma and dendritic processes of hippocampal neurons. Particularly, we were interested in understanding the differential contributions of iGluR and mGluR inputs for generating local Ca^{2+} and DAG signals and thus for inducing PKCγ translocation and activation. Our study shows that effective translocation of PKCγ in dendritic branches can only be triggered by combined local Ca^{2+} and DAG signals. Interestingly, dendritic DAG elevations could be generated by either mGluR-mediated activation of phospholipase C (PLC) or direct activation of a δ isoform of PLC by strong Ca^{2+} influx after NMDA receptor (NMDAR) or voltage operated calcium channel (VOCC) activation. In contrast, Ca^{2+} elevation could be sustained not only by such an influx but also by the release from intracellular Ca^{2+} stores, provided they had been replenished by preceding electrical activity.

Overall, our study indicates that PKCγ can play a central role in nonlinear processes typical of neuronal function by acting as a powerful coincidence detector of multiple signals, both synaptic and extrasynaptic. This feature might explain why neurons exposed to moderate electrical activity, but not those exposed to strong activity, require the participation of mGluRs to produce LTP.

Materials and Methods

Cell cultures. Primary cultures of hippocampal neurons were prepared according to Ryan and Smith (1995) from 2- to 3-d-old Sprague Dawley rats. The animal use procedures were approved by the Institutional Animal Use and Care Committee of the San Raffaele Scientific Institute. Briefly, after quick subdivision of hippocampi into small sections, the tissue was incubated into Hank's solution containing 3.5 mg/ml trypsin type IX (Sigma, St. Louis, MO) and 0.5 mg/ml DNase type IV (Calbiochem, La Jolla, CA) for 5 min. The pieces were then mechanically dissociated in a Hank's solution supplemented with 12 mM $MgSO_4$ and 0.5 mg/ml DNase IV. After centrifugation, cells were plated onto poly-ornithine-coated coverslips and maintained in MEM supplemented with 0.3% glucose, B27 supplement, 2 mM glutamax, 5% fetal calf serum, and 3 μM Ara-C (1-β-D-cytosine-arabinofuranoside) (Sigma). Cultures were maintained at 37°C in a 5% CO_2 humidified incubator, and used between 8 and 12 d after plating. If not specified, chemicals were from Invitrogen (Grand Island, NY).

Cloning of expression vectors and hippocampal neuron transfection. The full-length rat PKCγ and the C1 tandem domain of PKCδ (C1t) (Codazzi et al., 2001) were cloned (*Bgl*II/*Eco*RI) initially into the pEGFP-N2 vector with EGFP at the C terminal (Clontech, Palo Alto, CA), and then the EGFP-fusion constructs were subcloned (*Xho*I/*Not*I) into pBATmod vector (Schnurbus et al., 2002). The PH domain of human PLCδ1 (Stauffer et al., 1998) was cloned (*Bsr*GI/*Xba*I) into the pEGFP-C2 vector (Clontech).

The C1t was also inserted into a pBATmod plasmid after the EMCV IRES (taken from pIRES2-EGFP; Clontech) using the *Nco*I site (pBATmod-IRES-C1t-EGFP). The *Xba*I/*Not*I fragment was then inserted into pOPRSVI-1 (Schnurbus et al., 2002) to obtain pOPR-IRES-C1t-EGFP. The mouse PH domain of PLCδ4, obtained by PCR (sense, ATA GTC TAG ATG ACA TCT CAG ATT CAA GAC; antisense, GTA ACT CGA GTT AAT CCA CCA ACA GCT GGA GTC CT) from the EST clone with GenBank accession number BE655544 (Invitrogen), was then cloned (*Xba*I/*Xho*I) into pOPR-IRES-C1t-EGFP to obtain pOPR-PH-IRES-C1t-EGFP (reported as PH4-ires-C1t-EGFP in Results).

The plasmid transfection was performed by two different approaches, obtaining similar results in terms of cell viability. After 8–12 d in culture, the neurons were first infected with vaccinia virus (MVAT7pol; 20 min at 37°C) (for details, see Schnurbus et al., 2002), transfected with plasmids under T7 promoter by a polycationic lipid vector (Lipofectin; Invitrogen; 30 min at 37°C), and analyzed 12–18 h later. With the second approach, the cells were transfected with Lipofectamine 2000 (Invitrogen) and analyzed 24 h later.

TIRM and fura-2 videomicroscopy measurements. For Ca^{2+} measurements, cells were loaded for 20 min with fura-2 AM (Calbiochem) diluted in Krebs' Ringer's HEPES buffer (KRH) (containing 5 mM KCl, 125 mM NaCl, 20 mM HEPES, pH 7.4, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 6 mM glucose). The concentration of fura-2 AM was adjusted for each batch of the dye (typically from 2 to 4 μM) to the minimum value that gave reliable signals. When K^+ concentration was increased in the solution, the concentration of Na^+ was adjusted to maintain isotonicity. All experiments were performed at room temperature. Before each experiment, the coverslips were washed three times with KRH and the experiments were performed, where not explicitly indicated, in the same buffer.

The TIRM setup was built using a Zeiss Axioskope 2 microscope (Zeiss, Oberkochen, Germany), with the laser excitation beam (488 nm single line; 150 mW; Melles Griot, Taby, Sweden) entering through a single mode fiber from below the coverslip through a fixed dove prism (Melles Griot). The prism and the glass of the coverslip were coupled by immersion oil with the same refractive index (1.52), and the beam was totally internal reflected at the glass–water interface. The angle used for total internal reflection (~70°) produced an exponentially decaying field above the glass surface with a penetration depth of ~80 nm.

The 340 and 380 nm wavelengths for fura-2 excitation were provided by a Polychrome IV (Till Photonics, Martinsried, Germany) through the epifluorescence pathway.

Fluorescence images were collected by a cooled CCD videocamera (PCO Computer Optics, Kelheim, Germany). The Vision software (Till Photonics) was used to control the protocol of acquisition and to perform data analysis.

If not specified, the chemicals used for receptor stimulation or inhibition were from Tocris (Bristol, UK).

Field stimulation. Electrical stimulation was generated between two parallel platinum electrodes (3 mm apart) lying on the coverslip. The volume of KRH was reduced to barely allow water immersion of the objective lens (500 μl). Current pulses of 5 ms were generated by an electrical stimulator (Isostim A320; WPI, Sarasota, FL). The standard protocol was a 1-s-long train, or "burst," of 40 current pulses (i.e., 40 Hz). The intensity of the applied current was adjusted to match spontaneous Ca^{2+} activity or set up to maximum intensity (10 mA output). Stimulation was synchronized with the acquisition by the Vision software (Till Photonics).

Data analysis. The fluorescence values of both $[Ca^{2+}]_i$ (340/380 fura-2 measurements) and EGFP (monitored by TIRM) are expressed as fold increase with respect to basal level. For each set of experiments, the number of neurons analyzed and the percentage of responsive cells (if <100%) are indicated. The data obtained in responsive cells are expressed as mean of peak fold increase (f.i.) of 340/380 measurements and construct-EGFP fluorescence, ±SD and 99% confidence limits for the mean (c.l.m.). Assessment of the presence/absence of an effect was based on comparison of the amplitude of signal shifts, after the application of a stimulus, with the fluctuations of the baseline. The effect was considered to be present when the response amplitude exceeded three times the baseline root mean square departure (RMS). No statistical analysis was performed on qualitative data (i.e., the percentage of cells presenting an effect), because many unpredictable biological and technical causes may introduce differences among transfected cells, and the precision and reliability of the estimated percentage of cells presenting a clear-cut effect is of no relevance to the scientific questions at hand. All changes reported were statistically significant ($p < 0.01$, *t* test).

Results

We investigated the signaling steps leading to translocation and activation of PKCγ in hippocampal pyramidal neurons from 2- to 3-d-old rats, after activation of iGluRs and mGluRs. Neurons, maintained in culture for 8–12 d, were transfected with EGFP-conjugated constructs that were used as fluorescent biosensors for monitoring either PKCγ translocation or the production of DAG or IP_3. The translocation of these fluorescent probes between cytosol and plasma membrane was explored by TIRM, an

imaging technique that allows the observation of small changes in plasma membrane localization of these probes even within the thinnest neuronal processes. TIRM measurements were combined with fura-2 Ca^{2+} imaging.

Ca^{2+} influx is required for PKCγ translocation in resting hippocampal neurons

Acute exposure to the excitatory neurotransmitter glutamate (20–100 μM) induced rapid and sustained elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), along with strong and equally rapid translocation of PKCγ-EGFP from the cytosol to the plasma membrane, both in cell body and dendrites (Fig. 1*A*,*B*). Both responses were consistently observed ($n = 45$, neurons analyzed in separate experiments; 340/380, peak f.i., 2.26 ± 0.65; 99% c.l.m., 2.00–2.52; PKCγ-EGFP, 95%, f.i., 2.82 ± 0.73; 99% c.l.m., 2.53–3.11). Neither significant translocation nor Ca^{2+} signals were observed when Ca^{2+} was omitted from the extracellular medium ($n = 7$) (Fig. 1*C*), indicating that intracellular Ca^{2+} stores do not play a major role in the glutamate response of cultured neurons at rest (i.e., only exposed to the environmental conditions and not under the influence of specific pharmacological or electrical stimulation). This conclusion was supported by the evidence that administration of 3,5-dihydroxyphenylglycine (DHPG) (50 μM), a specific agonist of group I mGluRs (the family coupled to IP_3/DAG production), did not produce by itself any $[Ca^{2+}]_i$ change or PKCγ-EGFP translocation ($n = 12$) (Fig. 1*D*).

Because mGluR activation was not sufficient to induce PKCγ translocation, we investigated whether specific activation of NMDAR induces PKCγ translocation. When neurons were stimulated by high concentrations of NMDA (100–500 μM, in Mg^{2+}-free buffer), both $[Ca^{2+}]_i$ elevation and PKCγ-EGFP translocation were comparable with those observed after administration of the glutamate itself ($n = 21$; 340/380 f.i., 2.24 ± 0.94; 99% c.l.m., 1.66–2.82; PKCγ-EGFP f.i., 2.95 ± 0.73; 99% c.l.m., 2.5–3.4) (Fig. 1*E*). Subsequent administration of the NMDAR blocker APV (100 μM) triggered a rapid return of PKCγ-EGFP to the cytosol. Surprisingly, after this protocol of NMDA addition and APV blockade, administration of glutamate was able to quickly restore the two responses (Fig. 1*E*), suggesting that pathways other than NMDA-mediated Ca^{2+} entry also contribute to PKCγ translocation to the plasma membrane.

NMDAR stimulation directly promotes DAG production

Because PKCγ activation requires Ca^{2+} as well as DAG signals and because NMDA triggered marked PKCγ translocation, we investigated whether an increase in $[Ca^{2+}]_i$ was sufficient to also produce DAG. We used the DAG-binding tandem C1 domain from PKCδ (C1t-EGFP) as a fluorescent translocation indicator for DAG production. This TIRM-based approach is more sensitive than biochemical methods and can reveal spatiotemporal variations in individual live cells (Oancea et al., 1998; Codazzi et al., 2001). Administration of 1,2-dioctanoylglycerol (DiC8) (100 μM), a plasma membrane permeant analog of DAG, was used at the end of experiments to elicit maximal C1t-EGFP translocation (Fig. 2*A*). After exposure to 100 μM glutamate, DAG production was fast and transient ($n = 72$; 340/380 f.i., 2.12 ± 0.42; 99% c.l.m., 1.99–2.25; C1t-EGFP, 94%, f.i., 1.73 ± 0.33; 99% c.l.m., 1.63–1.83) (Fig. 2*A*). Unexpectedly, 500 μM NMDA (administered in Mg^{2+}-free buffer) also elicited a marked increase in plasma membrane C1t-EGFP fluorescence in a high proportion of the cells ($n = 60$; 340/380 f.i., 2.32 ± 0.83; 99% c.l.m., 2.03–2.61; C1t-EGFP, 52%, f.i., 0.4 ± 0.17; 99% c.l.m., 0.32–0.48) (Fig. 2*B*). The signal was observed both in soma and dendrites, frequently restricted to discrete regions along the shaft conferring a punctate appearance (Fig. 2*Bb*,*Bd*,*Be*). The increase in DAG production was often slightly delayed and with a slower onset, when

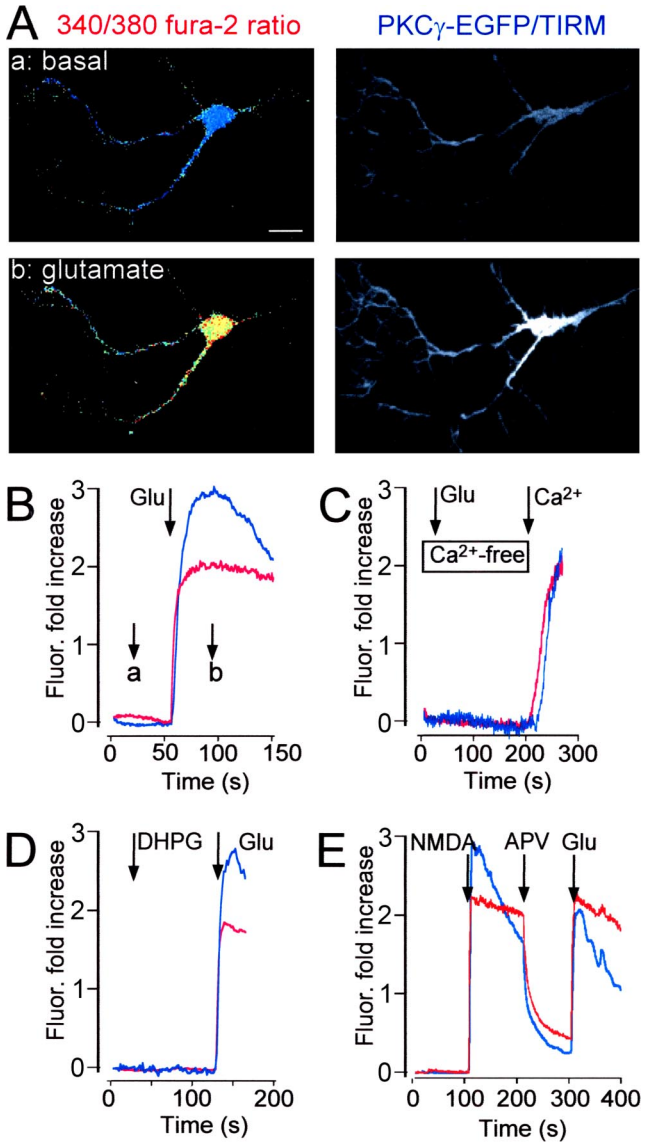

Figure 1. Ca^{2+} influx is required for glutamate-induced $[Ca^{2+}]_i$ elevation and PKCγ-EGFP translocation. In this and in the following figures: (1) the fluorescence values of both $[Ca^{2+}]_i$ (340/380 fura-2 measurements) and EGFP (monitored by TIRM at the plasma membrane in neurons transfected with the specified constructs) are expressed as fold increase above basal values; (2) $[Ca^{2+}]_i$ changes are represented by either a red line (in color figures) or a thin line (in black-and-white figures), whereas EGFP fluorescence values are represented by either a blue or a thick line; (3) when pseudocolor images (rainbow palette) are used, an increase in values is represented as a change from blue to red; (4) the exposure to pharmacological agents was continual after their administration (pointed by an arrow). ***A***, The two series of images show that glutamate administration (Glu) (20 μM) causes both an increase in $[Ca^{2+}]_i$ and translocation of PKCγ-EGFP from the cytosol to the plasma membrane in a pyramidal neuron. Scale bar, 20 μm. ***B***, The graph illustrates the temporal variations in $[Ca^{2+}]_i$ and PKCγ-EGFP translocation, measured in the cell body of the neuron in ***A*** (a and b refer to the corresponding pairs of images). ***C***, Administration of 20 μM glutamate in Ca^{2+}-free medium (containing 1 mM EGTA) fails to evoke a response. Reintroduction of extracellular Ca^{2+}, in the presence of glutamate, elicits both $[Ca^{2+}]_i$ response and PKCγ-EGFP translocation. ***D***, Neuron is responsive to glutamate (20 μM) but not to mGluR (DHPG; 50 μM) stimulation. ***E***, Administration of 500 μM NMDA (in Mg^{2+}-free KRH, containing 5 μM glycine) induces both a $[Ca^{2+}]_i$ increase and a PKCγ-EGFP translocation that are completely reverted by a NMDAR blocker (100 μM APV, in the presence of 1.2 mM Mg^{2+}). Under these conditions, subsequent stimulation with glutamate (20 μM) is able to restore the previous responses. Fluor., Fluorescence.

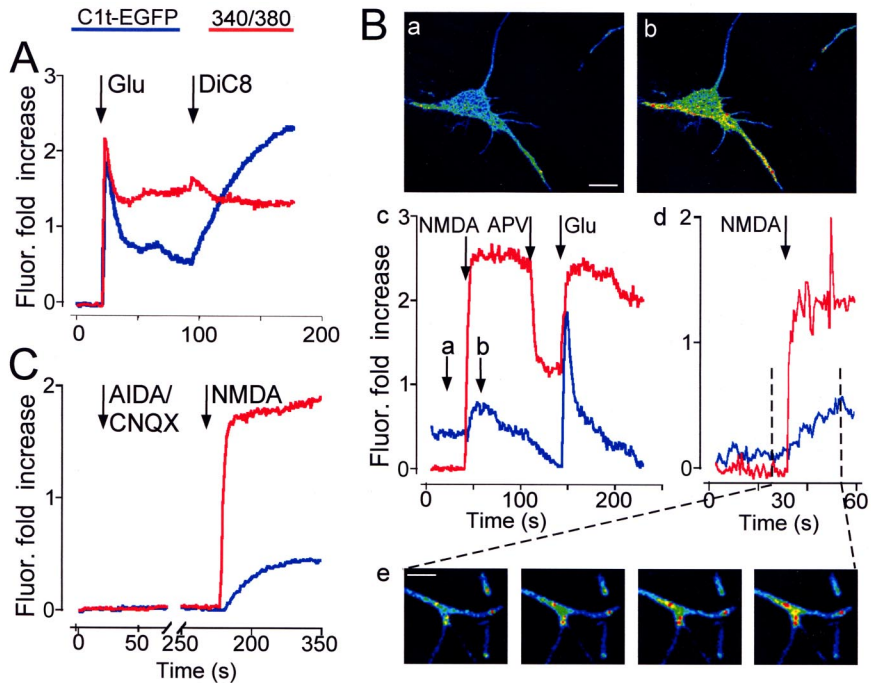

Figure 2. DAG production after glutamate and NMDA stimulation. ***A***, Administration of glutamate (20 μM) causes $[Ca^{2+}]_i$ elevation and rapid DAG production (as revealed by plasma membrane translocation of C1t-EGFP). Maximal translocation is reached after exposure to DiC8 (100 μM), a membrane-permeant analog of DAG. ***B***, The images ***a*** and ***b*** show C1t-EGFP signal in both soma and dendrites (punctate appearance) before and after NMDA (500 μM) stimulation. The graph ***c*** illustrates the changes in $[Ca^{2+}]_i$ and C1t-EGFP signal, measured in the soma of the above neuron exposed to the same protocol of stimulation of Figure 1*E* (the arrows a and b refer to the corresponding two images). Note that administration of APV causes a comparable decline in $[Ca^{2+}]$i and PKCγ translocation (Fig. 1*E*), whereas the decrease in DAG levels has slower kinetics. ***d*** illustrates the NMDA-mediated $[Ca^{2+}]_i$ elevation and C1t-EGFP translocation measured in the dendrite shown in the series of images in ***e*** (selected at regular intervals within the time window marked in ***d***). The pictures show the progressive increase in C1t-EGFP signal within hot spots. Scale bars: ***a***, 12 μm; ***e***, 3 μm. ***C***, Selective blockers of group I mGluRs (AIDA; 1 mM) and AMPA/kainate receptors (CNQX; 20 μM) do not prevent the NMDA-induced DAG production. Fluor., Fluorescence.

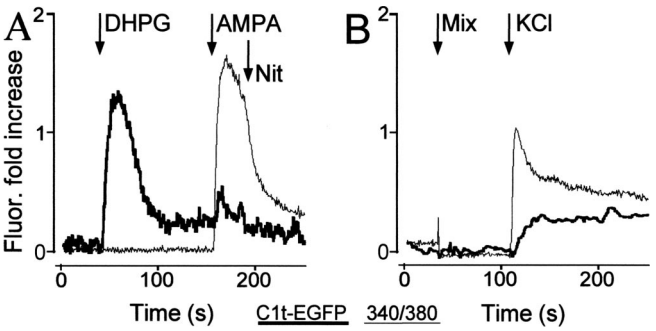

Figure 3. DAG production can be sustained by a non-NMDA Ca^{2+} influx. ***A***, The temporal analysis shows that DAG production is elicited by activation of both group I mGluR (DHPG; 50 μM) and AMPA receptor (AMPA; 100 μM) agonists. Note that stimulation of AMPA receptors, but not mGluRs promotes a concomitant $[Ca^{2+}]_i$ elevation, which is inhibited by nitrendipine (Nit) (10 μM). ***B***, A mix of glutamatergic receptor antagonists (100 μM APV, 20 μM CNQX, and 1 mM AIDA) does not prevent DAG production (C1t-EGFP translocation) after depolarization induced by KCl (30 mM). Fluor., Fluorescence.

compared with that observed in the same neuron after blockade of NMDARs and exposure to glutamate. The above observations led us to further investigate whether NMDA induces DAG production directly or indirectly. NMDA stimulation might promote DAG production as the result of a chain of events, involving activation of neurons, firing of action potentials, glutamate release from synaptic terminals, and, finally, activation of postsynaptic metabotropic receptors. This was not the case because NMDA was equally effective in inducing C1t-EGFP plasma membrane translocation even in the presence of 2 μM tetrodotoxin (a sodium channel blocker; $n = 8$; C1t-EGFP, 62%, f.i., 0.37 ± 0.17; 99% c.l.m., 0.16–0.58) (data not shown) or 1 mM 1-aminoindan-1,5-dicarboxylic acid (AIDA) plus 20 μM CNQX (two blockers of group I mGluRs and AMPA receptors, respectively; $n = 10$; C1t-EGFP, 50%, f.i., 0.38 ± 0.19; c.l.m., 0.18–0.58) (Fig. 2*C*).

Glutamate promotes DAG production via iGluR and mGluR pathways

Stimulation of mGluR with DHPG (50 μM) did not change $[Ca^{2+}]_i$ but induced a remarkable production of DAG ($n = 11$; C1t-EGFP f.i., 1.18 ± 0.32; 99% c.l.m., 0.87–1.49). Subsequent administration of a high concentration of AMPA (100 μM) caused a marked increase in $[Ca^{2+}]_i$ (340/380 f.i., 1.78 ± 0.52; 99% c.l.m., 1.28–2.28; sensitive to 10 μM nitrendipine, a VOCC blocker) accompanied by additional DAG production ($n = 11$; C1t-EGFP, 63%, f.i., 0.35 ± 0.13; 99% c.l.m., 0.12–0.58) (Fig. 3*A*). These experiments suggest that DAG is generated by activation of either mGluRs or iGluR through distinct mechanisms. In particular, AMPA receptors are known to increase $[Ca^{2+}]_i$ via activation of VOCCs (Marshall et al., 2003), which may in turn trigger DAG increases. In line with this possibility, also a depolarization induced by administration of high extracellular K^+ concentration (30 mM) to neurons in which both mGluRs or iGluRs were blocked (100 μM APV for NMDA, 20 μM CNQX for AMPA/kainate, and 1 mM AIDA for group I metabotropic glutamate receptors), produced a significant increase in C1t-EGFP translocation (more than five times the baseline RMS) in most neurons ($n = 20$; 70%). This suggests that the depolarization was not only effective in increasing $[Ca^{2+}]_i$ (340/380 f.i., 1.40 ± 0.72; 99% c.l.m., 0.94–1.86) but also able to promote a smaller but significant DAG production (C1t-EGFP f.i, 0.32 ± 0.17; 99% c.l.m., 0.18–0.46) (Fig. 3*B*).

We also tested whether calcium-induced calcium release via ryanodine receptors may contribute to the Ca^{2+} signals induced by Ca^{2+} influx. NMDA-induced DAG production was retained in neurons pretreated with both caffeine and ryanodine (20 mM and 10 μM, respectively), a standard protocol used to empty ryanodine-sensitive Ca^{2+} stores. Similarly, blockers of the calcium pumps responsible for store refilling did not alter Ca^{2+} responses (0.5 μM thapsigargin or 10 μM cyclopiazonic acid) (data not shown). This strongly suggests that, under these experimental conditions, Ca^{2+} entry across the plasma membrane is not amplified by Ca^{2+} release from intracellular Ca^{2+} stores.

Phospholipase Cδ is responsible for Ca^{2+}-induced DAG production

Among the various putative intracellular pathways by which Ca^{2+} signals might directly generate DAG, we focused on PLCδ,

an enzyme that has a calcium-stimulated catalytic activity 100-fold higher than that of the β- and γ-PLC isoforms (Rebecchi and Pentyala, 2000). To test this hypothesis, we first verified whether IP_3 was produced, along with DAG, after NMDA stimulation. The pleckstrin homology domain of PLCδ1 (PH1) was used as a sensor of IP_3 production (Hirose et al., 1999) and/or phosphoinositide 4,5-bisphosphate (PIP_2) hydrolysis (Stauffer et al., 1998). This PH1 domain binds to the polar head of PIP_2 and dissociates from the plasma membrane after PLC activation. When the fusion protein EGFP-PH1 was expressed in neurons, and fluorescence was monitored by TIRM, a clear decline of plasma membrane signal was observed (a sign of IP_3 production) along with the $[Ca^{2+}]_i$ increase induced by NMDA (n = 10; 340/380 f.i., 1.37 ± 0.87; 99% c.l.m., 0.48–2.26; PH1-EGFP f.i., −0.70 ± 0.14; 99% c.l.m., from −0.56 to −0.84) (Fig. 4*A*).

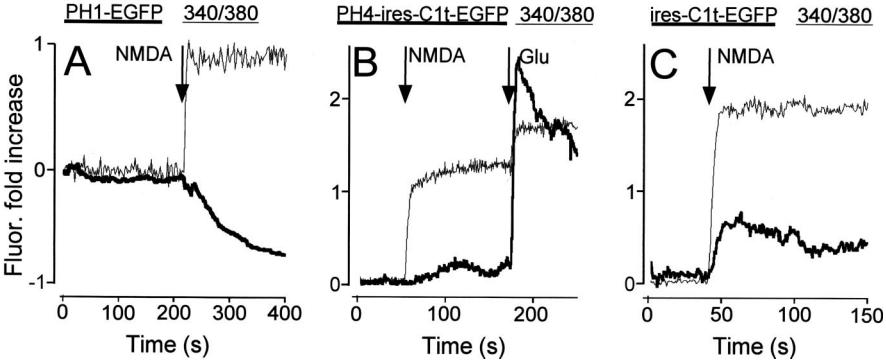

Figure 4. PLCδ activation after strong Ca^{2+} influx. ***A***, The involvement of a Ca^{2+}-dependent PLC was investigated in NMDA-stimulated neurons. The production of IP_3 was measured by monitoring translocation of the PH domain of the PLCδ1 (EGFP-PH1) from the plasma membrane to the cytosol (see details in Results). ***B***, A dicistronic vector was used for the concomitant expression of the inhibitory PH domain of PLCδ4 (PH4) and C1t-EGFP (DAG fluorescent biosensor). The overexpression of PH4-ires-C1t-EGFP virtually prevents the DAG production mediated by NMDA (500 μM) but not by glutamate (20 μM) stimulation. ***C***, A construct lacking the PH4 cassette (ires-C1t-EGFP) was used as a control. Overexpression of this construct induces the usual responsiveness to maximal concentration of NMDA. Fluor., Fluorescence.

To distinguish whether PLCδ or the β or γ isoform is activated, we overexpressed the N-terminal PH domain of PLCδ4 (PH4), which functions as a competitive inhibitor that blocks endogenous PLCδ (Nagano et al., 1999). The inhibitor PH4 domain was expressed in a dicistronic vector together with the C1t-EGFP domain, whereas it was omitted in an otherwise identical control construct (PH4-ires-C1t-EGFP dominant-negative construct and ires-C1t-EGFP control construct, respectively; see Materials and Methods).

The $[Ca^{2+}]_i$ responses to NMDA (500 μM in Mg^{2+}-free buffer) were not affected by the expression of either construct. In the PH4-ires-C1t-EGFP-transfected neurons (Fig. 4*B*), only a minority of the cells (n = 19; 16%) showed a translocation of C1t-EGFP, whereas in the ires-C1t-EGFP-transfected control neurons (Fig. 4*C*), the percentage of responses was maintained (n = 23; 70%). When we considered the mean response in the two populations, a 76% reduction was observed in neurons transfected with the inhibitory construct, consistently with the data reported by Nagano et al. (1999). Interestingly, glutamate administration (100 μM) retained its ability to stimulate C1t-EGFP plasma membrane translocation in cells transfected with PH4-ires-C1t-EGFP (n = 12) (Fig. 4*B*), suggesting that alternative PLC isoforms (presumably β) are exploited by mGluR.

Moderate iGluR stimulation promotes PKCγ activation only when coupled to mGluR stimulation

The above results suggest that different signaling pathways are integrated at the level of PKC. In a key test of this hypothesis, we investigated synergistic contributions between iGluRs and mGluRs to PKCγ activation. We first found an intermediate range of NMDA concentrations (5–10 μM) that was able to trigger maximal Ca^{2+} signals (n = 22; 340/380, 86%, f.i. = 1.95 ± 0.32; c.l.m., 1.66–2.24), although with a 2.2 times slower rise time, but was ineffective in stimulating C1t-domain translocation (n = 7) (Fig. 5*A*).

For these submaximal stimulation conditions, PKCγ-EGFP translocation was absent in 68% of cells and significantly reduced in the others (f.i., 0.39 ± 0.13, compare with 2.95 ± 0.73 reported above, referred to 500 μM NMDA) (Fig. 5*B*). This suggests that intermediate NMDAR activation triggers Ca^{2+} signals but not DAG production. Because we found previously that activation of mGluRs triggers DAG but not Ca^{2+} signals (Fig. 3*A*), PKCγ is in a central position to integrate these two signaling pathways. Consistent with such a mechanism, addition of DHPG (50 μM) to neuron prestimulated with intermediate NMDA concentrations now triggered rapid and marked PKCγ-EGFP translocation (n = 15; PKCγ-EGFP, 66%, f.i., 2.38 ± 0.34; c.l.m., 2.03–2.73) (Fig. 5*B*). Thus, the molecular control of PKC activation by Ca^{2+} and DAG provides for a coincidence detection mechanisms at the level of iGluR and mGluR activation with NMDAR triggering primarily Ca^{2+} signals and mGluRs triggering primarily DAG signals.

We also tested this form of synergy in a more physiological context. We took advantage of the fact that a minority of neurons displays spontaneous $[Ca^{2+}]_i$ transients of small amplitude; events can also be mimicked by electrical field stimulation. These small Ca^{2+} transients (n = 21; 340/380 f.i., 0.65 ± 0.26) did not lead to the production of DAG (data not shown) or significant translocation of PKCγ (Fig. 5*C*). When DHPG was administered to these neurons, the Ca^{2+} loaded into the stores by the preceding activity was released, giving rise to a Ca^{2+} transient comparable in amplitude with those previously elicited by the electrical activity. Under these conditions, the combination of Ca^{2+} and DAG signals now leads to PKCγ translocation (PKCγ-EGFP, 71%, f.i., 0.37 ± 0.17) (Fig. 5*C*). This provides evidence for an additional amplification mechanism by which mGluR stimuli not only produce DAG but can also contribute to Ca^{2+} signals. The subsequent superimposition of an electrical stimulus further reinforced Ca^{2+} elevation and PKCγ translocation (Fig. 5*C*).

The spatial changes elicited by this experimental protocol were investigated in dendrite branches, the region in which the highest synaptic activity is expected to occur. When a moderate Ca^{2+} influx was coupled with mGluR activation, PKCγ translocation was rapid and very high in small spots along the dendrites (Fig. 5*Df*). In contrast, PKCγ translocation was significantly delayed (2–3 s) and small in the regions in between these hot spots as well as in the soma (Fig. 5*Dg*). Finally, when an electrical stimulus of high intensity was applied, even in the absence of DHPG administration, $[Ca^{2+}]_i$ elevation was very high in the dendrites (although moderate in the soma) and PKCγ-EGFP translocation was observed to occur in hot spots along the shaft, conferring again a punctate appearance (Fig. 5*E*). Together, these experi-

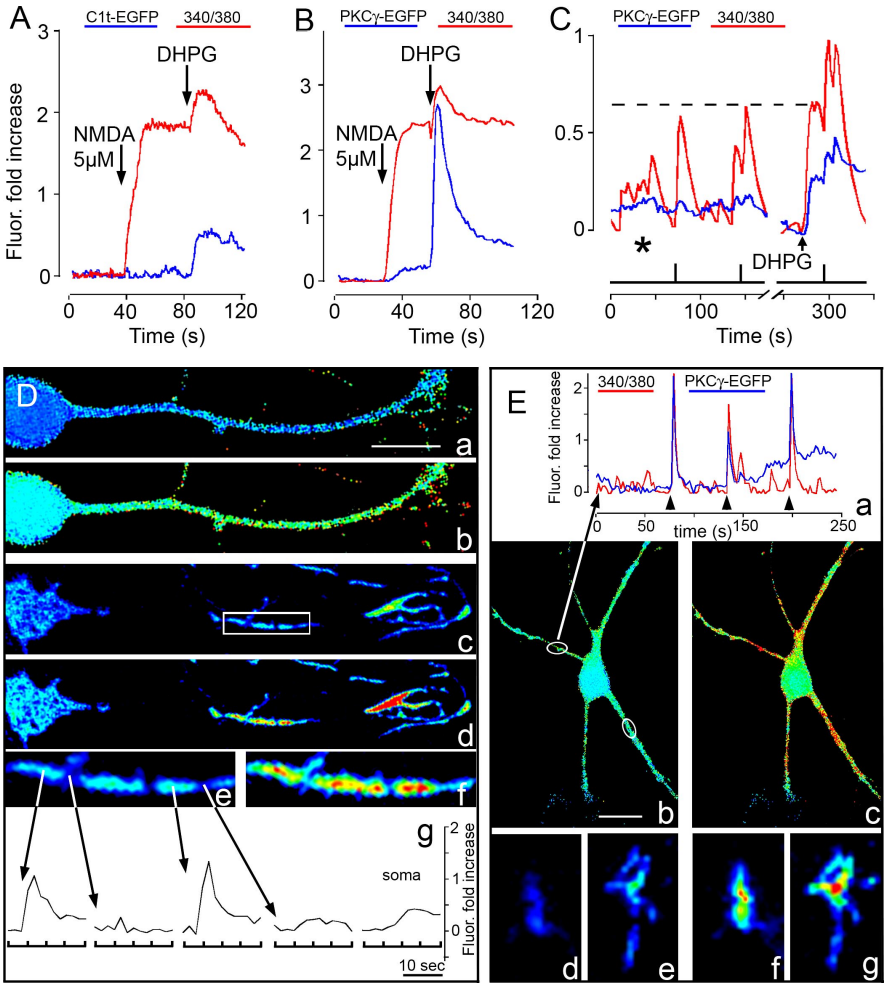

Figure 5. Cooperation between iGluRs and mGluRs in the activation of PKCγ. ***A***, Moderate stimulation of NMDARs (5 μM NMDA) causes a $[Ca^{2+}]_i$ increase characterized by a slow rising phase. This condition fails to promote DAG production (C1t-EGFP), which is promptly activated after subsequent stimulation of group I mGluRs (DHPG; 50 μM). ***B***, Changes in $[Ca^{2+}]_i$ elevation and PKCγ-EGFP translocation are shown after a protocol of stimulation as in ***A***. Concomitant activation of NMDARs and mGluRs appears to be required to promote efficient PKCγ-EGFP translocation. Note that, in ***A*** and ***B***, the $[Ca^{2+}]_i$ increase induced by NMDA stimulation favors intracellular store loading with ensuing Ca^{2+} discharge after DHPG challenging. ***C***, Small $[Ca^{2+}]_i$ increases, occurring spontaneously (*) or mediated by electrical field stimulation (the line below the graphs shows when pulses were applied), fail to promote a PKCγ-EGFP translocation in the absence of DHPG. In contrast, a DHPG-induced $[Ca^{2+}]_i$ response of comparable amplitude (see dashed line) proves effective. The subsequent electrical stimulus reinforces both $[Ca^{2+}]_i$ and PKCγ responses. ***D***, ***a***, ***c***, and ***e*** refer to a resting condition, whereas ***b***, ***d***, and ***f*** show the peak of the response to DHPG administered concomitantly with an electrical stimulus. The $[Ca^{2+}]_i$ variation (***a***, ***b***) is illustrated together with the PKCγ-EGFP fluorescence increase (***c***, ***d***). ***e*** and ***f*** highlight the dotted appearance of PKCγ-EGFP translocation in a portion of a dendrite (***c***, white box). The temporal analysis of the above experiment in various areas (***g***) draws attention to the higher amplitude and faster kinetics of PKCγ-EGFP translocation within the hot spots, compared with the interposed areas or the soma. Scale bar, 20 μm. ***E***, The graph in ***a*** shows that intense electrical stimulations (arrowheads) promote in the dendrites both high elevation of $[Ca^{2+}]_i$ (see ***b*** and ***c***) and PKCγ-EGFP translocation. The small figures (***d–g***) highlight PKCγ-EGFP translocation in two small areas of dendrites (***e*** and ***g*** vs ***d*** and ***f***, respectively). Scale bar, 25 μm. Fluor., Fluorescence.

ments suggest that mGluR stimuli and PKCγ activation are strengthened by previous electrical activity that leads to the loading of Ca^{2+} stores. Furthermore, the activation of PKCγ by combined electrical activity, NMDAR and mGluR signals is a local signaling event that is triggered primarily at punctate sites along the dendritic branches.

Discussion

Our study focused on the synergistic roles of iGluR and mGluR in the activation of PKCγ. We were able to gain new insights into this activation process by combining for the first time Ca^{2+} imaging with TIRM analysis of DAG and IP_3 production as well as PKCγ translocation in primary cultured hippocampal neurons. This allowed us to spatially resolve the production of these second messengers and to dissect the activation process of PKCγ.

Maximal activation of iGluRs is sufficient to promote DAG production and PKCγ translocation

We found that administration of high concentrations of glutamate to neurons leads to PKCγ translocation mainly as a consequence of Ca^{2+} influx. Neither IP_3- nor Ry-sensitive Ca^{2+} stores played a relevant role in this activation process. This was not unexpected because Ca^{2+} stores in neurons have been reported to be empty at rest and to replenish only when Ca^{2+} stores are filled by electrical activity (Shmigol et al., 1994; Rae et al., 2000). This condition is not specific of primary cultures, because also in unstimulated slices of hippocampus the intracellular stores were reported to act as powerful buffers, rather than regulated sources of Ca^{2+} (Garaschuk et al. 1997) or to provide a significant contribution only after they were loaded by a preceding exposure to high $[Ca^{2+}]_i$ (Rae and Irving, 2004). It was more surprising that Ca^{2+} influx, mediated either by NMDARs or VOCCs, did not only induce PKCγ translocation but also triggered the production of DAG. This is relevant for PKCγ, because its enzymatic activation requires both Ca^{2+} and DAG (Oancea and Meyer, 1998; Cho, 2001). We therefore tested whether the increase in $[Ca^{2+}]_i$ could directly stimulate PLC activation and DAG production in these neurons. Evidence for such a direct mechanism has been obtained in biochemical and cellular studies in insulin-producing cells, glial cells, and cerebellar neurons (Mizuguchi et al., 1991; Rebecchi and Pentyala, 2000; Codazzi et al. 2001; Okubo et al., 2001; Mogami et al., 2003). We now report that Ca^{2+} activation of the δ isoform of PLC is likely responsible for this response. This conclusion is based on the finding that overexpression of the N-terminal PH domain of the PLCδ4, a negative regulator of PLCδ (Nagano et al., 1999), prevented DAG production after NMDA stimulation. This construct is specific for PLCδ, because it did not block the mGluR-mediated activation of PLCβ under the same experimental condition. This indicates that maximal glutamate stimulation or depolarization elicits Ca^{2+} signals sufficient to activate PLCδ and thus to produce the necessary DAG for PKCγ activation. This Ca^{2+}-triggered production of DAG may explain how PKCγ can be activated during LTP protocols that do not involve coactivation of mGluRs (Wilsch et al., 1998).

Synergistic activation of PKCγ after iGluR and mGluR stimulation

The situation was different when neurons were exposed to intermediate stimulation protocols. For lower amplitude stimuli, for which electrical or NMDAR stimulation did not generate detect-

able levels of DAG, but still triggered near-maximal Ca^{2+} responses, we did not see significant translocation of PKCγ. This likely reflects the previous findings that PKC is only minimally activated in the presence of high $[Ca^{2+}]_i$ when DAG is not present (Oancea and Meyer, 1998). Interestingly, selective activation of type I mGluRs showed the opposite results, with marked production of DAG but with no significant Ca^{2+} signals generated. Accordingly, no significant translocation of PKCγ was observed after mGluR activation in resting hippocampal neurons. However, when submaximal activation of NMDAR was combined with mGluR stimulation, PKCγ became active as evidenced by a marked translocation to the plasma membrane (Fig. 6). Similar results were obtained with Ca^{2+} transients triggered by electrical field stimulation. Again, these Ca^{2+} signals were only effective in stimulating PKCγ when mGluRs were concomitantly activated.

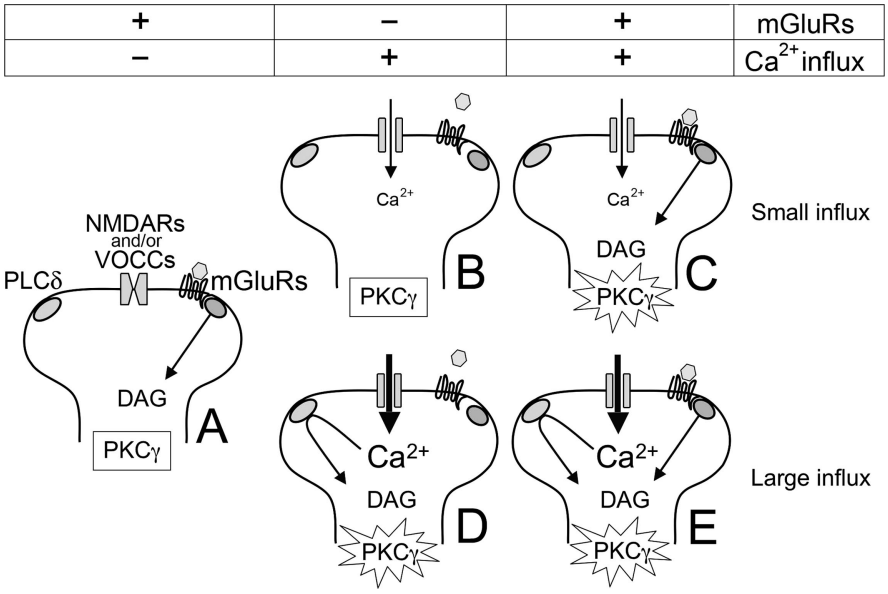

Figure 6. Scheme of the different pathways leading to PKCγ activation. $[Ca^{2+}]_i$ increase and DAG production are both required for PKCγ activation in synapses of hippocampal neurons at rest. Stimulation of group I mGluRs in resting synapses leads only to DAG production but not PKCγ translocation (***A***). Under this condition, no $[Ca^{2+}]_i$ elevation is produced, because the intracellular Ca^{2+} stores are functionally empty. A small Ca^{2+} influx is able to activate PKCγ only when mGluRs stimulation produces DAG (compare ***B***, ***C***). In contrast, a strong Ca^{2+} influx leads to PKCγ activation even in the absence of mGluRs activation, because it promotes DAG elevation via PLCδ (***D***, ***E***).

The role of mGluRs in synaptic plasticity has been mostly discussed as a means to release Ca^{2+} from IP_3-sensitive stores, leading to increases in $[Ca^{2+}]_i$ within the spine (Wilsch et al., 1998; Kotecha et al., 2003). This view reflects the widely accepted notion that the extent of $[Ca^{2+}]_i$ elevation represents a major determinant in the induction of synaptic plasticity (Lynch et al., 1983; Malenka and Nicoll, 1999; Lamprecht and LeDoux, 2004; Malenka and Bear, 2004). Our data confirm that intracellular stores are charged by electrical activity, and this is in agreement with the proposal that they contribute to synaptic function by representing a form of transient memory of the previous neuronal activity (Kovalchuk et al., 2000; Rae et al., 2000). However, our study provides evidence for a second and, arguably, more unique role for mGluR in that it triggers the production of DAG. Unlike the mGluR-triggered release of Ca^{2+} from stores, we found that the production of DAG is independent of whether or not the Ca^{2+} stores are filled and can thereby provide a physiological role for mGluRs even when stores are not loaded.

Together, these considerations suggest that the molecular requirement of PKCγ for Ca^{2+} and DAG is translated in neurons into a coincidence detection mechanism at the level of the upstream receptors. The required increase in cytosolic Ca^{2+} is primarily provided by Ca^{2+} influx through VOCC or NMDAR channels, and the required DAG signal is primarily generated by the activation of mGluRs. Our study also shows that this coincidence requirement can be overcome in two situations: first, when Ca^{2+} signals are sufficiently strong to also promote the activation of PLCδ and the production of DAG and, second, when mGluRs trigger IP_3-mediated Ca^{2+} signals in neurons whose Ca^{2+} stores were loaded by previous activity. It is tempting to draw a parallel between these molecular results and LTP induction. Under low stimulation conditions, coactivation of both NMDARs and mGluRs have been reported to be needed for LTP induction (Miura et al., 2002; Kotecha et al., 2003) and that PKC inhibitors but not IP_3 antagonists were shown to block LTP (Fujii et al., 2004).

Finally, we also found that electrically and mGluR-induced PKCγ translocation was markedly localized to microdomains along dendritic branches, whereas there was a much smaller and delayed translocation in between the spots and in the soma. These sites are not necessarily single synapses; rather they may represent dendritic "PKCγ signaling regions" where integration of signals from multiple synapses, via glutamate spillover from individual active boutons (Kullmann and Asztely, 1998), occurs. Alternatively, they may represent sites that respond to the glutamate released from stimulated adjacent astrocytes (Parpura et al., 1994).

A modified model for conventional PKC activation

Our finding that Ca^{2+} and DAG are both required in these neurons for effective PKCγ translocation also deserves additional attention. According to a recently proposed model, conventional PKC is driven to the plasma membrane and is partially activated by Ca^{2+} alone in the absence of DAG (Oancea and Meyer, 1998; Ron and Kazanietz, 1999). Here, we report that effective translocation of PKCγ in hippocampal neurons requires the presence of Ca^{2+} as well as DAG, suggesting that translocation is closely correlated with activation. In a model that takes the different previous results into account, the increase in cytosolic Ca^{2+} enhances the plasma membrane affinity of PKCγ (via its C2 domain), but the enhanced binding affinity is not strong enough to slow the shuttling of PKCγ between the cytosol and the plasma membrane. Only when DAG is generated will the weak interaction of PKCγ with the plasma membrane be strengthened, by now including not only the C2 domain but also the binding of one or both of the C1 domains with plasma membrane DAG. Only under these circumstances is PKCγ maximally translocated in the TIRM measurements and the enzyme in the conditions to be fully activated.

In conclusion, our main finding is that PKCγ, which requires Ca^{2+} and DAG for translocation and activation, functions as a coincidence detector for iGluR and mGluR stimulation. Whereas electrical and NMDAR stimuli preferentially generated Ca^{2+} and not DAG signals, mGluR preferentially generated DAG and not Ca^{2+} signals (Fig. 6). This clear distinction could be overcome for

two experimental conditions. First, in the case of maximal electrical or NMDAR stimuli, a Ca^{2+}-mediated activation of PLCδ was responsible for a direct production of DAG, enabling activation of PKCγ in the absence of mGluR stimulation. Second, in a process akin to molecular memory, electrical activity was found to load Ca^{2+} stores so that subsequent activation of mGluR triggered not only DAG production but also Ca^{2+} increases sufficient for PKCγ activation. Finally, high-resolution imaging of dendritic branches showed that PKCγ activation is a localized process that preferentially occurs in microdomains along the dendritic branches.

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