Intracellular Ca\(^{2+}\) store release contributes to activity-dependent synaptic plasticity in the central nervous system by modulating the amplitude, propagation, and temporal dynamics of cytoplasmic Ca\(^{2+}\) changes. However, neuronal Ca\(^{2+}\) stores can be relatively insensitive to increases in the store-mobilizing messenger inositol 1,4,5-trisphosphate (IP\(_3\)). Using a fluorescent biosensor we have visualized M\(_1\) muscarinic acetylcholine (mACH) receptor signaling in individual hippocampal neurons and observed increased IP\(_3\) production in the absence of concurrent Ca\(^{2+}\) store release. However, coincident glutamate-mediated synaptic activity elicited enhanced and oscillatory IP\(_3\) production that was dependent upon ongoing mACH receptor stimulation and S-\(\alpha\)-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid receptor activation of Ca\(^{2+}\) entry. Moreover, the enhanced levels of IP\(_3\) now mobilized Ca\(^{2+}\) from intracellular stores that were refractory to the activation of mACH receptors alone. We conclude that convergent ionotropic and metabotropic receptor inputs can facilitate Ca\(^{2+}\) signaling by enhancing IP\(_3\) production as well as augmenting release by Ca\(^{2+}\)-induced Ca\(^{2+}\) release.

There is now substantial evidence that fundamental neuronal properties ranging from membrane excitability through gene expression to regulation of synaptic plasticity can be modulated by changes in intracellular free calcium (Ca\(^{2+}\))/H\(_{11545}\). In particular, it has been increasingly recognized that neuronal intracellular stores, far from just acting as a sink for Ca\(^{2+}\), can play a critical role in modulating the amplitude, localization, propagation, and temporal dynamics of neuronal Ca\(^{2+}\), transients (1–3, 5, 6). The key current questions relate to the mechanisms that might control local and global Ca\(^{2+}\) signaling within the highly structured and functional domains of central neurons.

A major player in Ca\(^{2+}\) store release is the intracellular messenger IP\(_3\) that can be generated by phospholipase C-mediated hydrolysis of the minor membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP\(_2\))\(^{1}\). This signaling pathway is particularly prominent in the central nervous system, and a wide range of G protein-coupled receptors (GPCRs) can initiate the generation of IP\(_3\) in neurons (1). Currently, models support a role for IP\(_3\) receptors in the propagation of Ca\(^{2+}\) waves observed in dendrites following activation of GPCRs such as muscarinic acetylcholine (mACH) and metabotropic glutamate receptors (2, 7, 8). Moreover, Ca\(^{2+}\) influx via voltage-sensitive or receptor-operated Ca\(^{2+}\) channels can initiate dramatically synergistic Ca\(^{2+}\) release from intracellular stores in hippocampal and cortical pyramidal neurons by Ca\(^{2+}\)-induced Ca\(^{2+}\) release at IP\(_3\) receptors, providing potential ionotropic-metabotropic receptor coincidence detection signaling (7, 9–12). Alternatively or additionally, in view of the ability of Ca\(^{2+}\) influx to activate PLC activity in neurons (13–16) enhanced production of IP\(_3\) could further enhance regenerative Ca\(^{2+}\) release (7, 17). Indeed, the more favorable properties of IP\(_3\) rather than Ca\(^{2+}\) as a diffusible messenger (18) and the micro regional constraints imposed between the sites of generation and action of IP\(_3\) in neurons such as hippocampal CA1 pyramidal cells (7) or sympathetic ganglia (19) would support such a model.

In the present study we attempt to address these mechanisms by using a genetically expressed fluorescent biosensor for IP\(_3\) (the PH-domain of PLC-\(\delta\)). This probe binds PIP\(_2\) with high specificity and affinity (20) but translocates to the cytoplasm when IP\(_3\) accumulates (and PIP\(_2\) depletes) (16, 21–23). We have transfected this probe into cultures of rat hippocampal neurons and show a dramatic facilitation of IP\(_3\) production, which promotes Ca\(^{2+}\) store release. Synaptic activity within the neuronal network synergizes with mACH receptor activation to elicit enhanced and oscillatory IP\(_3\) production. Crucially, this augmentation of IP\(_3\) production mobilizes Ca\(^{2+}\) from stores that are refractory to the activation of mACH receptors alone.

**EXPERIMENTAL PROCEDURES**

*Hippocampal Cell Culture*—Rat hippocampal neurons from 1–3-day-old rat pups were grown in serum-free conditions using previously described methods (10, 24). Briefly, isolated hippocampi were cut into small pieces of about 2–3 mm\(^3\) and trypsinized for 30 min using 0.5 mg ml\(^{-1}\) Pronase E and 0.5 mg ml\(^{-1}\) thermolysin in a HEPES-buffered salt solution (HBSS) containing 130 mM NaCl, 10 mM HEPES, 5.4 mM KCl, 1 mM MgSO\(_4\), 25 mM glucose, and 1.8 mM CaCl\(_2\) at pH 7.2. Tissue pieces were then triturated in HBSS (Invitrogen) containing 40 mg ml\(^{-1}\) DNase, centrifuged and plated on 25-mm glass coverslips (3 per dish) at a density of 100,000 cells per coverslip. Cells were cultured in serum-free medium containing 60% Neurobasal medium (Invitrogen), 30% Dulbecco’s modified Eagle medium (DMEM), 5% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% sodium pyruvate, and 1% glutamine.

The abbreviations used are: PIP\(_2\), phosphatidylinositol 4,5-bisphosphate; GPCR, G protein-coupled receptor; mACH, muscarinic acetylcholine; PH, pleckstrin homology; IP\(_3\), inositol 1,4,5-trisphosphate; PLC, phospholipase C; GFP, green fluorescent protein; TTX, tetrodotoxin; NMDA, N-methyl-D-aspartic acid; VDCC, voltage-dependent Ca\(^{2+}\) channel; AMPA, \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid.

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pocampi) coated with 50 μg/ml 1poly-D-lysine. Initial growth medium was Neurobasal medium with B27 supplement (Invitrogen) containing 10% fetal calf serum to which was added 5 mM cytosine arabinoside after 24 h. At 72 h in vitro cells were grown in serum-free Neurobasal, and cultures were maintained for up to 4 weeks with 50% of the medium replaced every 3–4 days. Cultures were transfected at 5 days in vitro (DIV) and used for experimentation 2–3 weeks later.

**Plasmid Transfection—**Neurons were transfected with eGFP-PHPLC-δ (0.5 μg) using LipofectAMINE 2000 (3 μl per μg of DNA, Invitrogen). Briefly, to cells in Neurobasal media without antibiotics was added 100 μl of the transfection mix prepared in minimal essential medium (Invitrogen) per the manufacturer’s instructions and after 3–4 h the medium changed. This avoided the toxicity associated with LipofectAMINE. For experiments involving co-transfection of the truncated DsRed2-IP3 3-kinase a ratio of 2:1 to eGFP-PHPLC-δ was used (for details of constructs see Ref. 22). This produced nearly 100% co-transfection in test experiments using DsRed2 and eGFP (data not shown).

**Confocal Microscopy—**Translocation of eGFP-PHPLC-δ was visualized using an Olympus FV300 laser scanning confocal IX70 inverted microscope. Neurons were held at 37 °C using a temperature controller and microincubator (PDMI-2 and TC202A, Burleigh) and were perfused with HEPES buffered saline (HBS) containing 130 mM NaCl, 5.4 mM KCl, 1.0 mM MgCl2, 1.8 mM CaCl2, 10 mM HEPES, 10 mM glucose (pH 7.4). Images were captured using an oil immersion ×100 objective and ×4.5 optical zoom. Cellular IP3 levels were indexed as the relative change in fluorescence within a cytoplasmic region of interest as described previously (22, 23, 26). Drugs were applied (unless otherwise stated) through the perfusion lines at a rate of approx. 5 ml min⁻¹. Changes in intracellular Ca²⁺ concentration were determined in cells

![Image](https://example.com/image.png)

**FIG. 1. IP3 production in cultured hippocampal neurons visualized using GFP-PHPLC-δ translocation.** Hippocampal neurons in vitro were transfected with GFP-PHPLC-δ at 5 days in vitro as described under “Experimental Procedures” and maintained in culture for up to 4 weeks. Transfection efficiency was low; however, this allowed visualization of the extensive expression throughout the soma, dendritic tree, and spines of transfected neurons (A). Fluorescence enriched over the plasma membrane (B) because of the high affinity and select association of the pleckstrin homology domain of PLC-δ with PIP2. Perfusion with methacholine (100 μM) in the presence of 0.5 μM TTX to prevent action potential-mediated synaptic activity during the period indicated by the orange bar caused a reversible translocation to the cytoplasm of the cell soma (B) and the dendrites (C). The image pairs show the emission intensity (green) and its pseudocolor representation for the indicated regions of the neuron (A). The translocation of the biosensor indexed IP3 production rather than PIP2 depletion because maintaining static IP3 levels with a metabolic enzyme: IP3 3-kinase (orange), inhibited methacholine-stimulated translocation of GFP-PHPLC-δ (green, D). Graphical representations of the translocation of GFP-PHPLC-δ are shown in the presence of a kinase active or kinase dead version of IP3 kinase (E).
load with Fura4F-AM (1 μM, 1 h) using a Nikon Diaphot inverted epifluorescence microscope with an oil immersion objective (×40) and a Spectramaster II monochromator with a Merlin2000 data acquisition system (PerkinElmer Life Sciences). Cells were excited at 340 nm and 380 nm, fluorescence emissions were sequentially captured above 500 nm, and data were expressed as the 340:380 nm ratio, which is directly proportional to Ca2+. Dual IP3 and Ca2+ imaging was performed using the same system on cells transfected with eGFP-PHPLC Δ and loaded with Fura-4F.

Electrophysiology and Ca2+ Imaging—Simultaneous whole-cell current clamp recordings and Ca2+ measurements were made from neurons loaded with Fura4F-AM at 35–37 °C using thick walled glass pipettes (GC150F-7.5, Clark Electromedical, Reading, UK) with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), low-pass filtered at 5 kHz (8-pole Bessel filter), and sampled at 50 kHz. Cells were perfused with HBS and voltages recorded with pClamp 8.2 software (Axon Instruments). The intracellular solution contained 130 mM KCl, 10 mM HEPES, 5 mM EGTA, 1 mM MgCl2 and 40 mM sucrose. The pH was adjusted to 7.2 with KOH. Ca2+ dye was excited at 350 and 380 nm with a Polychrome II monochromator (T.I.L.L. Photonics, Martinsried, Germany). Emitted light was separated by a 400-nm dichroic mirror and filtered with a 420-nm long pass emission filter. Fluorescence ratios were acquired every 250 ms with a PentaMAX cooled charge-coupled device camera via a Gen IV image intensifier (Princeton Instruments, Trenton, NJ) and analyzed with MetaFluor software (Universal Imaging, West Chester, PA).

Data Analysis—Graphs and curve fitting of concentration-dependent data were plotted using Prism 3.0 (GraphPad Software Inc., San Diego, CA), and half-maximal (EC50) values were determined using the non-linear regression analysis available in the software. Data are presented throughout as representative traces and mean ± S.E. from at least three separate neurons and statistical comparisons made using Student’s paired or unpaired t test (p < 0.05 was considered significant). The images are presented as either the observed emission intensity (green) or as pseudocolor representations created using software available within the Olympus FV500 system. For quantitative analysis of data presented in Fig. 7, A–C, baseline levels of Ca2+ were taken as the average concentration prior to methacholine challenge in the presence of tetrodotoxin (TTx) or nominal [Ca2+] as, or the maximal peak height in the absence of TTx or the presence of Ca2+ as, and NMDA, respectively. This will slightly underestimate the actual contribution of synergetic IP3 production to Ca2+ changes. For the analyses of the effect of methacholine on picrotoxin-induced Ca2+ oscillations (see Fig. 7D) the mean of peak responses immediately before and after methacholine addition was compared. The data in Fig. 7E were tested using the analysis of co-variance (ANCOVA) package in Prism 3.0.

RESULTS

Initiation of IP3 Production by M1 mACH Receptor Activation—IP3 production was visualized in hippocampal neurons using a genetically expressed fluorescent biosensor, GFP-tagged pleckstrin homology domains of phospholipase C-δ1 (GFP-PHPLC Δ) (20–22). This enriched over the plasma membrane through its interaction with the IP3 head group of PIP2 (Fig. 1A). Activation of mACH receptors with methacholine (100 μM) caused a reversible translocation of the biosensor to the cytoplasm of the soma (Fig. 1B) and dendrites (Fig. 1C) in the presence of 0.5 μM TTx to block synaptic activity. This resulted from increased IP3 levels as opposed to IP3 depletion because metabolism of IP3 by co-expression with a DsRed2-tagged IP3 3-kinase A, but not the kinase-dead enzyme (22, 24), inhibited GFP-PHPLC Δ translocation by >80% (Fig. 1, D and E, n = 11, p < 0.001). Translocation following methacholine addition was also suppressed by co-transfection with a catalytically inactive form of G protein-coupled receptor kinase 2, which sequesters activated Goα11 subunits (25). These data demonstrate that

FIG. 2. Muscarinic acetylcholine receptor activation of IP3 production in hippocampal neurons. Concentration-dependent changes in GFP-PHPLC Δ were observed in response to challenge with agonist (A). This confirms that IP3 production in response to GPCR activation is graded within hippocampal neurons. In addition to methacholine, a cholinesterase-insensitive compound, carbachol, and one lacking affinity for nicotinic acetylcholine receptors, oxotremorine-M, elicited translocation of the biosensor (B). Carbachol, a non-selective mACH receptor agonist, fully inhibited translocation and antagonism of the response to 3 μM methacholine by pirenzepine at 300 nM suggesting involvement of the M1-subtype (C). When neurons were exposed to mACH receptor agonist in the absence of TTx unusual patterns of methacholine-induced IP3 production were often observed (D).
Gq/11 protein-coupled mACh receptors stimulate IP3 production in hippocampal neurons.

The pharmacological profile was consistent with the involvement of the M1 mACh receptor-subtype, which is the predominant form in the hippocampus coupled to Gq/11-activated IP3 production (Fig. 2) (25). Fig. 2A shows the IP3 responses to challenges with increasing concentrations of the cholinesterase-resistant mACh receptor agonist carbachol. A clear concentration-dependent increase in cytoplasmic fluorescence was observed indicating that GPCR-stimulated IP3 production is graded in single cells. This finding confirms earlier work showing graded IP3 production in simple cells (26). Plotting the peak responses for three different agonists reveals the typical sigmoid shape, with EC50 values that are consistent with the sole involvement of mACh receptors (Fig. 2B). Increases in IP3 were also completely inhibited by the mACh receptor antagonist atropine (Fig. 2C). At a concentration selective for M1 receptors (27), pirenzepine was able to block methacholine-induced IP3 production (Fig. 2C) further implicating the involvement of this subtype.

**Network Synaptic Activity Augments IP3 Production**—The above study was conducted in the presence of TTx, which prevents the synaptic activity that is commonly associated with the networks of “mature” hippocampal cultures (28–30). Interestingly, in some initial experiments conducted in the absence of TTx spikes in IP3 production above the mACh receptor-stimulated levels were sporadically observed during sustained agonist challenge (Fig. 2D). Phenotypically these changes contrasted markedly with the oscillatory patterns of IP3 production recorded in non-excitable cells (21–23, 31). It seemed unlikely, therefore, that these involved either the dynamic uncoupling mechanism described for metabotropic glutamate-R5a receptor-induced IP3 oscillations (22, 31) or the smaller Ca2+/H11001-induced IP3 oscillations observed for α1B-adrenergic receptors (23). However, because fluctuations in IP3 concentration were only evident in hippocampal cultures challenged with mACh receptor agonist in the absence of TTx a synergy between action potential-induced synaptic activity and Gq/11-coupled GPCR activation was indicated. To test this hypothesis the synaptic activity within the hippocampal cultures was studied.

Simultaneous recordings of membrane potential and Ca2+/H11001 revealed spontaneous synaptic firing that correlated with bursts of action potentials and concurrent Ca2+ oscillations (Fig. 3B). These occurred synchronously in all hippocampal neurons suggesting the formation of a neural network. Oscillations were prevented by the AMPA/kainate receptor antagonist CNQX (20 μM) and removing extracellular Ca2+/H11001 but were relatively unaffected by NMDA receptor inhibition with d-AP5 (50 μM) (D).
network (34), reproducibly induced similar synchronous bursts of action potentials and oscillatory Ca\(^{2+}\) changes (Fig. 3, A and C). These Ca\(^{2+}\) oscillations were inhibited by TTX (Fig. 3B) and occurred simultaneously in all neurons in a field of view (Fig. 3C, inset). The AMPA/kainate antagonist CNQX (20 \(\mu M\)) and removal of extracellular Ca\(^{2+}\) prevented the oscillatory changes in Ca\(^{2+}\) observed in hippocampal neurons, but these were only slightly reduced in amplitude by an NMDA receptor antagonist d-AP5 (50 \(\mu M\), Fig. 2D).

Incubating hippocampal neurons with picrotoxin alone failed to influence IP\(_3\) levels, but upon the addition of methacholine (30 \(\mu M\)) large amplitude oscillations in IP\(_3\) production were observed (Fig. 4A). Conversely, challenge with methacholine elicited stable IP\(_3\) production that was dramatically enhanced after the addition of picrotoxin and inhibited by TTX (Fig. 4B). Importantly, continuous mACH receptor activation was required for the oscillatory enhancements to continue indicating the necessity for GPCR-PLC coupling (Fig. 4A). Although we routinely recorded IP\(_3\) in the cell soma the same phenomenon also occurred in the dendrites (Fig. 4, C and D). The synergistic IP\(_3\) synthesis was inhibited by treatments that blocked AMPA/kainate receptors and voltage-dependent Ca\(^{2+}\) channels (VDCCs) but not NMDA receptors (Fig. 5). Fig. 5A shows a representative experiment where methacholine (30 \(\mu M\))-induced increases in IP\(_3\) were potentiated by perfusion with picrotoxin (100 \(\mu M\)). This was inhibited in a transient manner by the co-application of CNQX (20 \(\mu M\)), a selective AMPA/kainate channel antagonist. In contrast, application of the NMDA channel antagonist was without effect (Fig. 5B). Removing extracellular Ca\(^{2+}\) also inhibited the oscillatory potentiation of IP\(_3\) production (Fig. 5C). Similarly, application of CdCl\(_2\) (100 \(\mu M\)), a non-selective VDCC antagonist, inhibited the synergism between synaptic activity and mACH receptor activation (Fig. 5D). The potentiating action of coincident synaptic activity was clearly shown when concentrations of methacholine that failed to elicit IP\(_3\) increases were found to significantly increase upon application of picrotoxin. The continuous activation of mACH receptors was again shown to be important because synergistic IP\(_3\) production was lost by perfusion with atropine even in the continued presence of synaptic activity (Fig. 5F).

These data reveal the strong synergy between activation of the M\(_1\) mACH receptor and synthetically driven, AMPA receptor-mediated depolarization and Ca\(^{2+}\) entry through VDCCs. Recent studies by Okubo et al. (16) in cerebellar Purkinje cells using an identical biosensor revealed that Ca\(^{2+}\) influx following activation of AMPA receptors could stimulate IP\(_3\) production. These authors suggested the involvement of the Ca\(^{2+}\)-activated PLC-\(\delta\) family. In contrast, in the present study it should be emphasized that for hippocampal neurons Go\(_{q/p1}\)-activated PLC isoforms (most likely PLC-\(\beta1\) and/or PLC-\(\beta4\)) are an absolute prerequisite for the enhancement of IP\(_3\) production.

A Post-synaptic Ca\(^{2+}\)-dependent Augmentation of IP\(_3\) Production—Repetitive AMPA application (10 \(\mu M\) for 1 s) mimicked the augmentation of mACH receptor-induced IP\(_3\) production in neurons by synaptic activity (Fig. 6A) and potentiated methacholine-stimulated responses by 500\% (methacholine response \(\pm 10 \mu M\) AMPA, \(n = 8\), \(p < 0.005\)). This strongly implies that the interaction is at a post-synaptic level rather than a
modulation of glutamate release following changes in network excitability. We also determined whether the enhancement of mACH receptor-induced IP3 production was specific to AMPA receptors or the result of elevated Ca2+ levels. Although NMDA channels play little or no role in the synaptic activity-mediated potentiation of IP3 production, application of NMDA increased Ca2+ levels in the hippocampal neurons. Challenging neurons with NMDA alone had little effect on IP3 production; however, co-application with methacholine potentiated IP3 production (Fig. 6B; mean amplification 1000%, n = 14, p < 0.001). In combination the data suggest that synaptic activity in hippocampal cultures elicited glutamate release to activate AMPA receptors, which depolarized neurons to activate VDCCs. This resulted in an increase in Ca2+ that synergized with mACH receptor activation to lead to synergistic IP3 production. Because action potential-induced synaptic activity occurs as bursts in hippocampal neuronal cultures this resulted in oscillatory IP3 increases. The ability of NMDA to mimic the action of AMPA indicated that it is the increase in Ca2+ that mediated the potentiated responses.

**Enhanced IP3 Production Is Accompanied by Mobilization of Ca2+ Stores**—Next we ascertained the functional significance of the enhanced IP3 production on Ca2+ store release. When neurons presenting pre-existing network activity, manifest as synchronous repetitive Ca2+ spikes, were perfused with TTx, activity ceased and the Ca2+ declined to a lower basal level (Fig. 7A). When these cells were challenged with methacholine (30 μM) for 30 s in the continued presence of TTx, increases in Ca2+ were rarely observed (Fig. 7A, 9 of 101 cells). However, upon TTx removal network activity restarted and methacholine routinely elicited Ca2+ increases (Fig. 7A, 90 of 101 cells). The peak Ca2+ responses above the background levels were significantly greater (0.112 ± 0.008 ratio units) during neural network activity than in its absence (0.019 ± 0.002). Perfusion with nominally Ca2+-free buffer, which similarly prevented network activity (Fig. 7B), also inhibited methacholine-elicited...
Ca\textsuperscript{2+} increases (10 of 73 versus 70 of 73 cells in the presence of Ca\textsuperscript{2+}). Peak responses to methacholine (100 µM) were 10-fold greater in the presence of Ca\textsuperscript{2+} (0.222 ± 0.016 ratio units) compared with responses in the absence of Ca\textsuperscript{2+} (0.022 ± 0.003). The synergism did not result from network activity per se because perfusion with NMBA in the presence of TTX raised Ca\textsuperscript{2+} levels and facilitated methacholine-induced Ca\textsuperscript{2+} release (Fig. 7C). Peak methacholine responses were 0.138 ± 0.007 and 0.048 ± 0.004 (mean ± S.E., n = 93, p < 0.001) in the presence and absence of NMBA, respectively. The enhanced Ca\textsuperscript{2+} responses were a consequence of Ca\textsuperscript{2+}-store mobilization, because they did not occur following pre-incubation with the Ca\textsuperscript{2+}-ATPase inhibitor cyclopiazonic acid to deplete the intracellular Ca\textsuperscript{2+}-stores (Fig. 7A, inset).

Methacholine (30 µM) also significantly increased (10.7 ± 0.5%; n = 169, p < 0.001) the amount of Ca\textsuperscript{2+} observed during picrotoxin-induced network oscillations (Fig. 7D). Plotting the peak increase observed in the presence of methacholine against that in its absence revealed a linear relationship that was offset but parallel to the line of equality (Fig. 7E, p > 0.05 for comparison of slope, and p < 0.001 for y axis intercept point compared with line of equality). This suggests that under these conditions the synergism between mACH receptor activation and synaptic activity reaches a threshold required to release Ca\textsuperscript{2+} from the stores irrespective of the background Ca\textsuperscript{2+}. Dual IP\textsubscript{3} and Ca\textsuperscript{2+} imaging experiments revealed that initiating network activity with picrotoxin and subsequent perfusion with methacholine resulted in oscillatory spikes of IP\textsubscript{3} underlying the enhanced Ca\textsuperscript{2+} spikes (Fig. 7D). Importantly, synergistic IP\textsubscript{3} production was required, because without methacholine (but in the continued presence of increased cellular IP\textsubscript{3} levels) the enhancement was no longer observed (Fig. 7D).

Methacholine also enhanced AMPA-mediated increases in Ca\textsuperscript{2+} (Fig. 7F). Rapid application of AMPA (100 ms) elicited reproducible peaks in Ca\textsuperscript{2+} that were significantly increased (47 ± 4%, mean ± S.E., 96 cells, p < 0.001) during periods of perfusion with methacholine (30 µM).

**DISCUSSION**

In many studies activation of G\textsubscript{q/11}-coupled GPCRs alone failed to initiate release from Ca\textsuperscript{2+} stores in neurons (7, 9, 19). Although the relative insensitivity of neuronal IP\textsubscript{3} receptors (35) or Ca\textsuperscript{2+} store filling (10, 12) may contribute, it has recently been demonstrated that the proximity of the components of the PLC signaling pathways could be crucial to Ca\textsuperscript{2+} release. In studies on sympathetic neurons (19, 36), GPCR-mediated elevations in IP\textsubscript{3} only promoted release of Ca\textsuperscript{2+} from stores when they occurred rapidly and close to the IP\textsubscript{3} receptors. When levels increased intervals relatively slowly, such as by diffusion from distant sites of synthesis, no Ca\textsuperscript{2+} release occurred because of a rapid feedback inhibition of the IP\textsubscript{3} receptors by Ca\textsuperscript{2+}/calmodulin. In the excitatory neurons of the hippocampus IP\textsubscript{3} receptors, in contrast to ryanodine receptors, appear to be located extrasynaptically on the dendritic shafts (2). Thus, any IP\textsubscript{3} generated at synapses on spines must diffuse from its site of production to its site of action. In addition, the IP\textsubscript{3} 3-kinase “firewall” juxtaposed to the cytoplasmic face of the plasma membrane limits the diffusional capacity of IP\textsubscript{3} in hippocampal neurons (24). However, the facilitated IP\textsubscript{3} synthesis that we have identified in this study might be expected to have a major contribution to these characteristics of neuronal Ca\textsuperscript{2+} store release. A rapid amplification of IP\textsubscript{3} production could provide a means to achieve the requisite rapid accumulation to open the IP\textsubscript{3} receptors. Furthermore, the amplification of IP\textsubscript{3} production could significantly enhance the ability of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release to activate more of the cellular population of IP\textsubscript{3} receptors. Although it is well established that the activation of IP\textsubscript{3} receptors is under the control of both IP\textsubscript{3} and Ca\textsuperscript{2+} (4) the sequence of binding of these ligands is crucial. Initial binding of IP\textsubscript{3} reveals the stimulatory Ca\textsuperscript{2+} site facilitating more Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release, whereas initial Ca\textsuperscript{2+}-binding inhibits the IP\textsubscript{3} receptor (37).

Our data indicate that although Ca\textsuperscript{2+} stores in hippocampal neurons are largely refractory to mACH receptor activation alone, coincident synaptic activity and synergistic IP\textsubscript{3} production facilitate Ca\textsuperscript{2+} release. The synergy is dependent upon both Ca\textsuperscript{2+} entry and G\textsubscript{q/11} activation and could provide both positive feedback and potential coincidence detection. This appears to differ from the situation in cerebellar Purkinje cells in which AMPA receptor-stimulated, voltage-dependent Ca\textsuperscript{2+} influx results in enhanced IP\textsubscript{3} production (16), although it is difficult to eliminate a constitutive GPCR activation in those studies. Our own data would suggest that at least initially, a G\textsubscript{q/11}-activated PLC-β is involved in this response, but whether there is recruitment of a separate Ca\textsuperscript{2+}-dependent form of PLC, such as PLC-δ, remains to be established. A recent study has revealed that Ca\textsuperscript{2+} entry-dependent PLC-γ2 translocation and activation plays a key role in the amplification of IP\textsubscript{3} production in B lymphocytes (38). Because this enzyme is expressed in neuronal cells (39), it represents a further potential target for feed-forward activation in hippocampal neurons.

Overall, the current results strongly support a model in which dramatically enhanced, Ca\textsuperscript{2+}- dependent IP\textsubscript{3} production either activates a relatively insensitive IP\textsubscript{3} receptor, or in which IP\textsubscript{3} itself acts as a mobile messenger to overcome spatial or catabolic barriers between the mACH receptor–G\textsubscript{q/11}, PLC complex and appropriate IP\textsubscript{3} receptors. Within neuronal spines this synergism could potentially allow IP\textsubscript{3} to mobilize Ca\textsuperscript{2+} from stores at dendritic junctions and would be consistent with
observations (7, 8) in which activation of synaptic ionotropic and metabotropic receptor signaling pathways leads to a focal rise in dendritic Ca\textsuperscript{2+} concentration that may be propagated to the soma invading the nucleus (40) potentially to initiate or regulate gene transcription.

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Synaptic Activity Augments Muscarinic Acetylcholine Receptor-stimulated Inositol 1,4,5-Trisphosphate Production to Facilitate Ca \(^{2+}\) Release in Hippocampal Neurons

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