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Temporal Synaptic Tagging by I_h Activation and Actin: Involvement in Long-Term Facilitation and cAMP-Induced Synaptic Enhancement

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Summary

Presynaptic I_h channels become activated during a tetanus through membrane hyperpolarization resulting from Na^+ accumulation and electrogenic Na^+/K^+ exchange. I_h activation is obligatory for inducing long-term facilitation (LTF), a long-lasting synaptic strengthening. cAMP-induced synaptic enhancement also requires I_h activation, and both processes are sensitive to actin depolymerization. Other mechanisms are responsible for expression of the responses. Once initiated, continued response to cAMP is I_h and actin independent. Moreover, LTF-induced activation of I_h renders subsequent cAMP enhancement insensitive to both I_h blockers and actin depolymerization. This actin-stabilized "temporal synaptic tagging" set by I_h activation is prolonged when I_h is activated concurrent with an elevation in presynaptic calcium concentration ($[Ca^{2+}]_i$), permitting the further strengthening of synapses given appropriate additional stimuli.

Introduction

Crustacean neuromuscular junctions display a long-term facilitation (LTF) of synaptic transmission following sustained intense activity of the motor neuron. This form of synaptic plasticity, lasting several hours (Atwood et al., 1975), or 1–2 days (Lnenicka and Atwood, 1985), is unusual in that its locus is entirely presynaptic, with "silent" synapses on motor neuron terminals becoming activated following tetanic stimulation (Wojtowicz and Atwood, 1985, 1986, 1988; Wojtowicz et al., 1988). A Ca^{2+}- and calcineurin-dependent induction of protein synthesis is required for the maintenance of LTF, which is apparently initiated by the coordinated activation of phostphatidylinositide-3-kinase (PI3 kinase), MAP kinase, and the rapamycin-sensitive kinase mTOR/FRAP (Beaumont et al., 2001). These properties distinguish LTF from other forms of long-term synaptic plasticity, and it is important to understand the mechanisms underlying its induction. Here we delineate the major steps in the initiation of LTF and describe a unique form of metaplasticity, which we call "temporal synaptic tagging," in which induction of LTF not only strengthens synaptic transmission but also alters the nature of synaptic responses to extrinsic stimuli that elevate cAMP concentration.

Previous work suggests that Na^+ influx during tetanic stimulation is necessary for LTF induction (Atwood et al., 1975), while other reports have suggested that LTF is induced by means of an ion-independent, voltage-dependent switch (Wojtowicz and Atwood, 1988), as well as by cAMP generation (Dixon and Atwood, 1989). Here we investigate the possibility that the "switch" necessary for induction of LTF is the activation of hyperpolarization-activated cation channels, or I_h channels, which are present in the presynaptic membrane of the crayfish exciter axon (Beaumont and Zucker, 2000). Supporting this hypothesis, our results indicate that block of I_h prior to LTF induction prevents subsequent LTF. We find that I_h becomes activated during a prolonged tetanus in response to membrane hyperpolarization. This develops through presynaptic Na^+ accumulation during tetanic stimulation, resulting in the increased activation of the Na^+/K^+ ATPase and an electrogenic exchange current that hyperpolarizes the presynaptic membrane.

We investigated the mechanism by which I_h activation results in synaptic enhancement. Our results indicate that activation of I_h, either through cAMP generation or directly via hyperpolarization, results in the generation of a transient synaptic marker or "tag"—whose generation is sensitive to disruption of the actin cytoskeleton. These tagged synapses increase transmitter output by subsequently responding to additional stimuli that would otherwise be ineffective. Furthermore, the lifetime of this tag appears to be significantly prolonged if I_h activation occurs concomitant with elevated presynaptic $[Ca^{2+}]_i$, allowing tagged synapses to later respond to stimuli applied at least 1 hr after the time the tag was set, even in the presence of inhibitors that block responses to such stimuli at untagged synapses. This "temporal synaptic tagging" represents a form of metaplasticity, in which LTF can alter the rules governing subsequent modulation of synaptic transmission by extrinsic signals.

Results

To test for the involvement of I_h channels in LTF induction, we use two drugs that specifically block this channel, ZD7288 and DK-AH 269. We have previously tested the potency of ZD7288 against crayfish I_h by characterizing its dose-dependent block of the presynaptic depolarization induced by forskolin-induced cAMP activation of I_h channels (Beaumont and Zucker, 2000). We now introduce DK-AH 269 (Janigro et al., 1997; Pape, 1994; Raes et al., 1998) as a blocker of crayfish I_h and characterize its pharmacological profile.

I_h Block Using DK-AH 269

Addition of DK-AH269 (3 nM–10 μM) following application of forskolin (30 μM; EC_{50} 19 ± 10 μM) potently reversed the 8.5 ± 0.5 mV cAMP-induced depolarization

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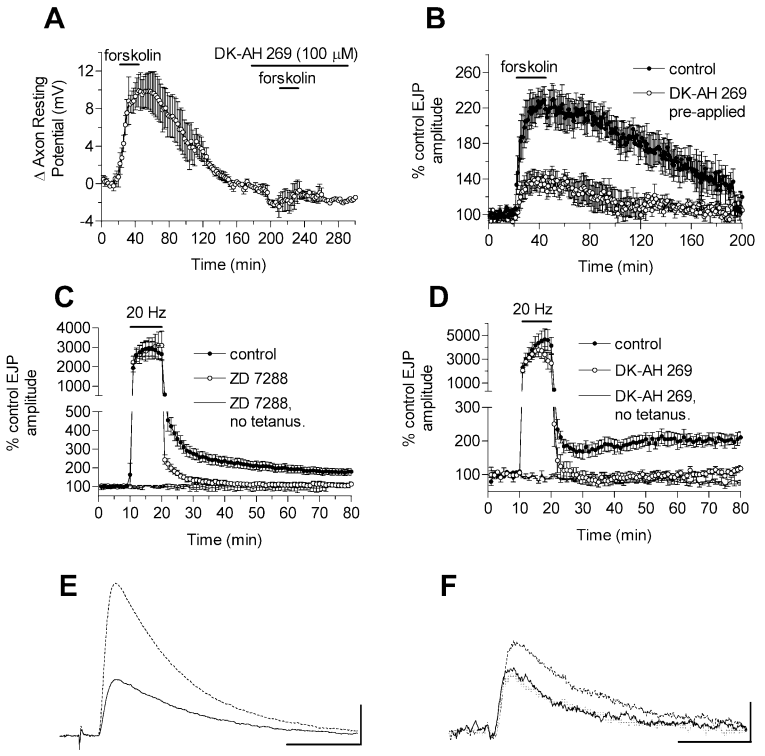

Figure 1. Presynaptic I_h Channel Activation Is Essential for Generation of cAMP Responses and LTF

(A and B) Characterization of DK-AH 269 as a suitable blocker of crayfish I_h channels. (A) Preapplication of the use-dependent I_h channel blocker DK-AH 269 (100 μM) was sufficient to abolish forskolin (30 μM)-induced axon depolarization. (B) DK-AH 269 (100 μM) reduced cAMP-induced synaptic enhancement by ~70%.

(C and D) Induction of LTF was achieved by a 20 Hz, 10 min tetanus, and the resultant synaptic enhancement lasted throughout the recording period (closed circles). Incubation with either of the I_h channel blockers ZD7288 (30 μM, [C]) or DK-AH 269 (100 μM, [D]), bath applied 30 min prior to and throughout the time shown, abolished LTF (open circles). ZD7288 (C) or DK-AH 269 (D) applied 30 min prior to and throughout the recording in the absence of tetanic stimulation caused no significant change in transmission recorded at 2 Hz (solid line with no symbols).

(E) Representative traces of excitatory junction potentials (EJPs) recorded at 2 Hz prior to an LTF-inducing tetanus (solid line) and 30 min posttetanus (dotted line) to show LTF. Scale bars, 0.25 mV/25 ms.

(F) EJPs recorded at 2 Hz before (solid line) and after a 30 min incubation with ZD7288 (30 μM) (thick gray line) show that ZD7288 did not significantly affect basal transmission. The EJP recorded 30 min following a 20 Hz LTF-inducing tetanus (dotted line) in ZD7288 showed reduced LTF expression. Scale bars, 0.05 mV/25 ms.

from the resting membrane potential of −71 ± 1 mV, with an IC_{50} of 125 ± 30 nM (n = 4). If DK-AH 269 was applied prior to forskolin application, when most I_h channels were closed, an approximately 10-fold higher concentration of DK-AH 269 was required (IC_{50} = 1.35 μM). These results are consistent with the previously reported use dependence of DK-AH 269 (Raes et al., 1998). Nevertheless, Figure 1A illustrates that a 100 μM DK-AH 269 preapplication was sufficient to result in a total block of forskolin-induced depolarization.

cAMP-induced activation of presynaptic I_h plays a critical role in cAMP-mediated synaptic enhancement at the crayfish NMJ (Beaumont and Zucker, 2000). We therefore expected that block of I_h by DK-AH 269 (100 μM) would significantly reduce forskolin-induced enhancement of synaptic transmission. Figure 1B shows that a 30 min application of forskolin resulted in a 120% ± 16% enhancement in transmission, which slowly reversed back to baseline levels following washout of forskolin. However, in the same preparations, if DK-AH 269 was applied for 30 min prior to and during a second forskolin application, the resultant enhancement was reduced to only 36% ± 11% (n = 5), identical to results previously reported following I_h block using ZD7288 (Beaumont and Zucker, 2000).

I_h Activation Is Essential for Induction of LTF

We have shown previously (Beaumont and Zucker, 2000) that I_h activation enhances synaptic transmission at crayfish neuromuscular junctions. Since tetanic stimulation induces a hyperpolarization that could activate I_h (Wojtowicz and Atwood, 1985), it seemed plausible that I_h activation is required for LTF induction. After establishing baseline amplitudes of excitatory junction potentials (EJPs) from proximal muscle fibers in response to a 2 Hz axonal stimulus, long-term facilitation was elicited following a 20 Hz, 10 min tetanic train (Figures 1C–1E). During the tetanus, EJP amplitude was elevated by the Ca^{2+}-dependent processes of facilitation, augmentation, and potentiation (Zucker, 1999). Immediately following the tetanus, EJP amplitude decayed biexponentially (τ_{decay1} = 11.5 ± 0.5 s, τ_{decay2} = 7.1 ± 0.9 min, n = 9), reflecting augmentation and posttetanic potentiation (PTP), to an average EJP amplitude greater than that observed pretetanus, and this was attributed to LTF. Decay of facilitation was not detected because it occurred too quickly to be captured by our posttetanic EJP sample rate of 2 Hz. LTF was distinguished from PTP by measuring EJP enhancement after complete decay of PTP at 20–60 min posttetanus, when LTF increased EJP amplitude by 113% ± 17% (n = 16).

To examine the involvement of I_h in LTF induction, either ZD7288 (30 μM; Figure 1C) or DK-AH 269 (100 μM; Figure 1D) was applied for 30 min prior to and during the 90 min LTF protocol. Both drugs blocked tonically active I_h, causing a 5.6 ± 1.1 mV (n = 6) and 5.1 ± 0.37 mV (n = 4) presynaptic hyperpolarization, respectively (see Figure 2). This hyperpolarization did not affect the

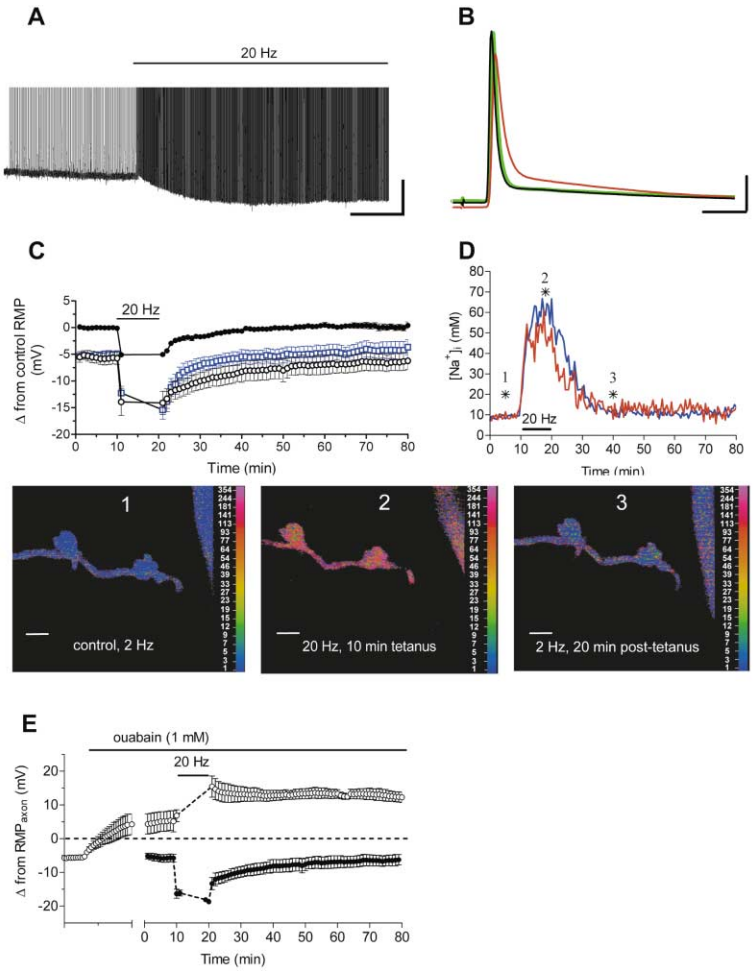

Figure 2. Membrane Hyperpolarization Resulting from Electrogenic Na^+/K^+ Exchange Activates I_h Channels during an LTF-Inducing Tetanus

(A) Stimulation of the exciter axon at 20 Hz resulted in membrane hyperpolarization, which developed to a maximum within 1 min. Action potentials were truncated. Scale bars, 10 mV/30 s.

(B) Representative action potentials recorded at 2 Hz prior to tetanic stimulation (black line), 1 min posttetanus (red line), and 40 min posttetanus (green line). Scale bars, 20 mV/10 ms.

(C) Time course of the change in axon resting membrane potential plotted throughout LTF induction and expression (closed circles) and following I_h channel block with ZD7288 (30 μM; open circles) or DK-AH 269 (100 μM; blue squares).

(D) Time course of sodium accumulation in boutons (red line) and primary/secondary branches (blue line) in response to a 20 Hz, 10 min tetanus (each trace is average of n = 4). (1–3) Representative ratiometric color images of SBFI fluorescence in two en-passant terminals and a tertiary branch: at 2 Hz axon stimulation (1), at 20 Hz (2), and then 20 min posttetanus at 2 Hz (3). Color bars indicates intracellular sodium concentration (mM) corresponding to a given color. Scale bar, 10 μm.

(E) After I_h channel block with ZD7288 (30 μM), a 20 Hz tetanus resulted in a large axonal hyperpolarization (closed circles) that was temporally correlated with axonal sodium accumulation and decay. Block of the Na^+/K^+ ATPase with ouabain (1 mM, 30 min prior to tetanus and throughout the experiment; open circles) resulted in axon depolarization, and prevented tetanus-induced hyperpolarization in the presence of ZD7288.

enhancement of EJP amplitude during tetanic stimulation (Figures 1C and 1D). However, little or no LTF could be induced after I_h block—transmission increased only 11% ± 11% (n = 8) in ZD7288 and 0.6% ± 10% (n = 6) in DK-AH 269. This is significantly less ($p < 0.005$) than the 98% ± 20% (n = 14) or 100% ± 19% (n = 6) enhancement during control LTF recorded from the same batches of crayfish used for respective experimental treatments. Control recordings made in the presence of I_h blockers but in the absence of tetanic stimulation (Figures 1C and 1D) showed that basal transmission was sometimes gradually reduced by DK-AH 269 (−19% ± 10%, n = 3) or ZD7288 (−4% ± 15%, n = 3). However, these modest, inconsistent, and insignificant reductions could not account for the much larger effects of these drugs following LTF induction. Our data demonstrate that synaptic enhancement during LTF is dependent on I_h channel activity.

An earlier study (Dixon and Atwood, 1989) reported LTF to be sensitive to the cyclase inhibitor SQ 23566, suggesting a role for cAMP in LTF generation. However, we found no effect of this drug, injected to a presynaptic concentration of 250 μM with 0.5 mM also present externally, on either LTF or the cAMP-dependent enhancement of synaptic transmission induced by forskolin (Beaumont and Zucker, 2000). SQ 23566 is apparently ineffective against crayfish adenylyl cyclase. We find, therefore, no evidence for any role of cAMP in LTF, although we cannot exclude this as a possibility. Our previous work also found no role for protein kinase A (PKA) in LTF (Beaumont et al., 2001) or cAMP-enhancement of transmission (Beaumont and Zucker, 2000).

I_h Is Activated during a Tetanus by Increased Na^+/K^+ ATPase Activity

We next investigated whether a hyperpolarization-dependent activation of I_h may underlie LTF. Indeed, axonal hyperpolarization (1 min, −30 nA current injection) sufficient to activate I_h was shown previously to induce EJP enhancement (Beaumont and Zucker, 2000). During an LTF-inducing tetanus, axons hyperpolarized by 5.1 ± 0.31 mV from a resting membrane potential of −70 ± 1.0 mV during the first minute of tetanic stimulation (n = 8; Figures 2A–2C). Action potentials broadened during the tetanus and the area under the voltage trace increased (Figure 2B) such that at a stimulation frequency of 20 Hz, membrane potential did not return to rest between action potentials, and accurate measurement of resting potential was difficult during the tetanus. However, immediately following the tetanus at 2 Hz stimulation, the resting potential remained hyperpolarized by 5.0 ± 0.37 mV, at which point axonal poten-

tial recovered to pretetanic levels within ~14 min (Figure 2B). This membrane hyperpolarization results from activation of the electrogenic Na^+/K^+ ATPase (3 Na^+ out: 2 K^+ in) in response to an increased intracellular Na^+ load (Wojtowicz and Atwood, 1988). After block of I_h by either ZD7288 (30 μM) or DK-AH 269 (100 μM), the hyperpolarization elicited by a tetanus approximately doubled to 9.8 ± 2.8 mV (n = 4) and 10.5 ± 2.1 mV (n = 4), respectively (Figure 2C), due to removal of the depolarizing drive caused by activation of the nonspecific cation-selective I_h channels.

We considered an alternative interpretation of the increased tetanic hyperpolarization caused by I_h inhibitors: block of tonically open I_h channels would remove a conductance pathway that shunts the membrane, and elimination of this shunt would result in a larger hyperpolarization to an outward exchange current. Using an equivalent RC circuit model of the presynaptic membrane, we estimated the contribution of I_h conductance to resting membrane conductance as ≤25% (calculated from the resting potential and with the assumption that the reversal potential for I_h is no more depolarized than −20 mV [see Pape, 1996]). Loss of this conductance removes a shunt during tetanic stimulation that should increase membrane hyperpolarization by only ~1.25 mV. The much larger increase in hyperpolarization observed on blocking I_h cannot be explained by this simple elimination of a shunt. Rather, it implicates further activation of I_h during the tetanus to counteract the tetanic hyperpolarization. This activation of I_h is responsible for the induction of LTF.

The dependence of the hyperpolarization on intracellular Na^+ load and Na^+/K^+ pump activity was demonstrated by measuring Na^+ accumulation during the LTF-inducing tetanus with the ratiometric dye sodium-binding benzofuran isophthalate (SBFI). In response to a 20 Hz, 10 min tetanus, $[Na^+]_i$ increased in axon branches by 51 ± 8 mM (n = 4) and in presynaptic boutons by 41 ± 11 mM (n = 4) from a resting level of 9.1 ± 2.4 mM. Figure 2D shows the time course and extent of the $[Na^+]_i$ increase, which corresponded closely to the time course of axonal hyperpolarization (Figure 2C). Additionally, axonal hyperpolarization in the presence of ZD7288 (30 μM) in response to a 20 Hz, 10 min tetanus was blocked by the Na^+/K^+ ATPase inhibitor ouabain (1 mM; Figure 2E). In its place, a depolarization appeared, probably as a consequence of posttetanic changes in intracellular Na^+ and extracellular K^+ concentrations.

Actin Dependence of I_h-Triggered Enhancement

The presynaptic regulation of cytoskeletal elements, especially actin, has been shown to affect transmission at some synapses (e.g., Cole et al., 2000; Kuromi and Kidokoro, 1998; Morales et al., 2000; Wang et al., 1996). Actin has been proposed to act as a "glue" or "brake," tethering reserve pools of vesicles away from active zones and allowing their regulated release following high-frequency activity, to increase the releasable vesicle pool, and subsequently increase transmitter output (Ceccaldi et al., 1995; Hilfiker et al., 1999; Humeau et al., 2001). Actin has also been proposed to act as a "track," delivering vesicles via associated kinesin and myosin-actin interactions to release sites (Bi et al., 1997). As both cAMP-dependent synaptic enhancement (Wang and Zucker, 1998) and LTF (Wojtowicz and Atwood, 1986; Wojtowicz et al., 1988, 1994) are manifested as an increase in total vesicle pool size, we investigated whether the actin cytoskeleton played any role in I_h-dependent synaptic enhancement. We treated preparations for 30 min before a tetanus and throughout the experiment with one of the structurally distinct actin depolymerizers swinholide A (10 μM, n = 5), cytochalasin D (10 μM, n = 5), or latrunculin B (3 μM, n = 6) (Spector et al., 1999). Figures 3A–3C show substantially diminished LTF, to 32 ± 22%, 40 ± 30%, and 59 ± 10%, respectively, from a control level of 115% ± 39% (n = 8). Actin disruption selectively reduced LTF, as basal synaptic transmission was unaffected. In contrast to the reduction in LTF expression, elevation of EJP amplitude during short-term plasticity was enhanced in the presence of all the actin depolymerizers, perhaps due to the removal of an actin "barrier" to release (Morales et al., 2000) or to activity-dependent mobilization of vesicles from an actin-tethered reserve pool (Trifaró and Vitale, 1993; Vitale et al., 1995; Wang et al., 1996).

In addition to the reduction of LTF expression, forskolin (30 μM)-dependent synaptic enhancement measured by 2 Hz stimulation was also significantly reduced by treatment with actin depolymerizers (n = 5 each; Figure 3D), suggesting that both forms of I_h-dependent synaptic enhancement rely on an intact presynaptic actin cytoskeleton.

Actin depolymerization could act either upstream or downstream of I_h channels. We found that actin disruption had only modest effects on the cAMP-dependent modulation of I_h. Cytochalasin D and Swinholide A reduced the axonal depolarization to forskolin (30 μM), compared to depolarization in the absence of these drugs (average reduction of 52% ± 11% and 24% ± 2%, respectively, n = 5 each treatment; Figure 3E). Latrunculin B had no effect on cAMP modulation of I_h (1% ± 7% change from control, n = 5). Our finding that the reduction of forskolin-induced synaptic enhancement in all cases was larger than the effect on I_h, suggests that actin acts mainly at a step subsequent to I_h activation.

LTF and cAMP-Dependent Enhancement Are Nonocclusive

Thus far, our results indicate that cAMP-dependent enhancement and LTF critically depend on both I_h activation and an intact actin cytoskeleton. We thus investigated whether activation of one form of synaptic enhancement could occlude the other. Figure 4A shows that a 57 ± 4% enhancement (n = 6) of EJP amplitude following LTF induction did not even partially occlude the response to a subsequent application of either forskolin (30 μM) or 8-Br cAMP (300 μM), which resulted in further 83% ± 14% (n = 3) and 74% ± 12% (n = 3) increases in EJP amplitude respectively (Figures 4A and 4B). This is no different ($p > 0.5$) from the 102% ± 34% or 80% ± 12% enhancement with forskolin (n = 5) or 8-Br cAMP (n = 5) seen in LTF-naïve preparations (Figure 4B). Including KT5720 (1 μM) in the bath failed to affect either the magnitude of LTF (157% ± 5%, n = 4, KT5720 absent, versus 153% ± 11%, n = 2, KT5720 present) or the EJP enhancement by forskolin or 8-Br

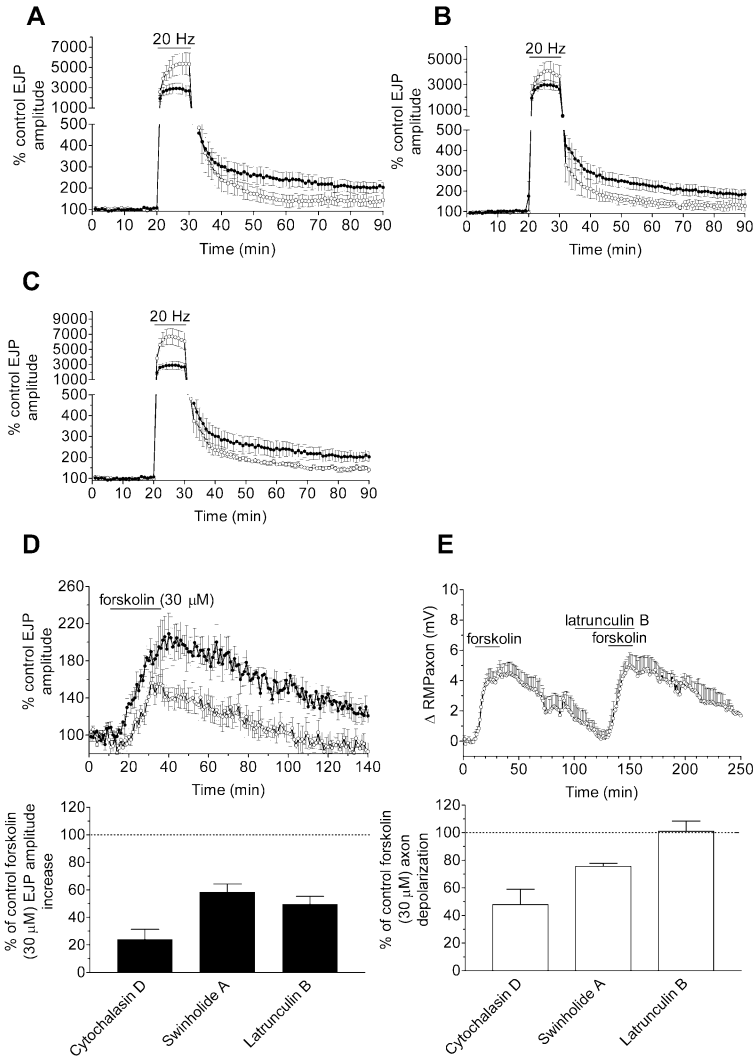

Figure 3. Disruption of Actin Interferes with I_h-Dependent Forms of Synaptic Enhancement

(A–C) LTF was reduced after incubation 30 min prior to and throughout the time shown with the actin depolymerizers (open circles) cytochalasin D (10 μM, [A]), swinholide A (10 μM, [B]) and latrunculin B (3 μM, [C]), compared to control responses (closed circles; [A–C]).

(D) Forskolin-induced synaptic enhancement was also depressed by actin depolymerization. The top panel shows the effect of incubation with latrunculin B (3 μM) on forskolin enhancement of synaptic transmission (open circles) compared to control forskolin enhancement (closed circles). The lower panel summarizes the reduction of synaptic enhancement to forskolin in the presence of different actin depolymerizers.

(E) The action of the actin depolymerizing drugs had variable effects on cAMP modulation of I_h channels. The top panel shows no effect of incubation with latrunculin B (3 μM) on forskolin depolarization of axon membrane potential compared to controls without depolymerization. The lower panel summarizes the reduction of forskolin-induced axon depolarization in the presence of different actin depolymerizers. Cytochalasin D and, to a lesser extent, swinholide A seemed to partially disrupt cAMP-induced channel modulation, but latrunculin B had no effect, suggesting that actin depolymerization with these agents predominantly affects processes downstream of I_h channel activation mediating synaptic enhancement.

cAMP (117% ± 10%, KT5720 absent, versus 112% ± 5%, KT5720 present), confirming the PKA-independent nature of LTF and the cAMP-dependent response.

This lack of occlusion is also seen if the experiment is performed in reverse order: a prior forskolin application resulting in a synaptic enhancement of 124% ± 38% did not occlude subsequent LTF induction (Figure 4C). The clarity of this result is somewhat confounded by the appearance of an initial posttetanus depression (cf. Wang and Zucker, 1998), most likely resulting from the excessive release (almost double control values) that occurs during the tetanus in the presence of forskolin. Nevertheless, after normalization of the pretetanic response in forskolin to 100% (Figure 4D), the posttetanus (20–60 min) EJP amplitude in the presence of forskolin was enhanced by 41% ± 9% ($n = 5$), not significantly different from the 57% ± 16% increase ($n = 10$) recorded during LTF in the absence of forskolin in paired controls ($p > 0.15$).

I_h Activation and an Intact Actin Cytoskeleton Are Not Required for Maintenance of cAMP-Dependent Synaptic Enhancement

The necessity for both I_h activation and actin in both LTF and cAMP-dependent enhancement, but the lack of occlusion between these forms of plasticity, suggests the possibility that other pathways are responsible for their maintenance. We therefore tested whether I_h activation and an intact actin cytoskeleton were necessary only in the induction phase of cAMP-dependent enhancement. This is already implied for LTF by the relatively transient activation of I_h during only the inducing tetanus (see Figure 2).

EJP amplitude was first elevated by forskolin, which was followed by coapplication of either ZD7288 (30 μM) or DK-AH 269 (100 μM) for 60–80 min to block I_h (Figures 5A and 5B), or latrunculin B (3 μM; Figure 5C) to induce actin depolymerization. Under these circumstances, application of either I_h blocker or the actin depolymerizer failed to affect the amplitude of cAMP-dependent synaptic enhancement. This result demonstrates that cAMP-induced activation of I_h is critical for induction, while other (cAMP-dependent) processes are responsible for maintenance. In addition, the integrity of the actin cytoskeleton is essential only during the induction, or I_h-dependent phase of enhancement. In three of the seven experiments shown in Figures 5A and 5B, the specific PKA inhibitor KT5720 (1 μM) was continuously present throughout the recording. No differences in responses (204% ± 31% maintained enhancement) were seen

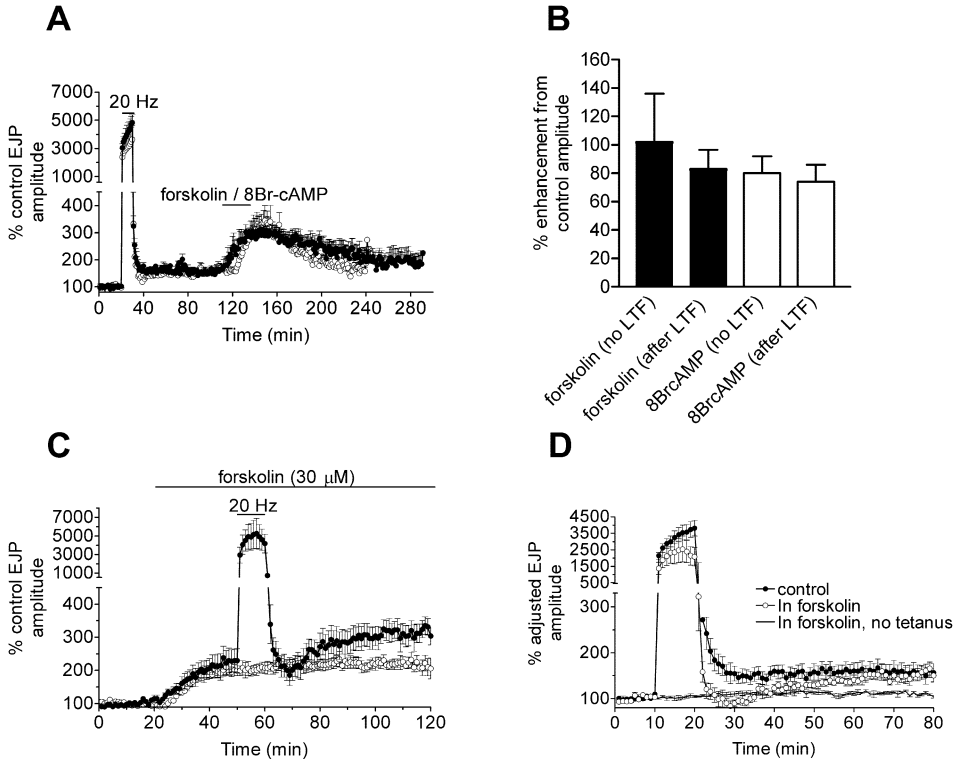

Figure 4. LTF and cAMP-Dependent Enhancement Are Mutually Nonocclusive

(A) Induction of LTF fails to occlude subsequent cAMP-dependent enhancement elicited either by forskolin (30 μM; closed circles) or by 8-Br cAMP (300 μM; open circles).

(B) Comparison of the cAMP response in LTF-naïve preparations, and following LTF induction, demonstrates that LTF failed to occlude cAMP-dependent enhancement.

(C) Application of forskolin approximately doubled EJP amplitude, reaching a plateau enhancement after ~25–30 min. The continued presence of forskolin failed to occlude subsequent LTF expression after delivery of a 20 Hz, 10 min tetanus (closed circles). Open circles show control recordings of forskolin applied for the same period in the absence of tetanic stimulation.

(D) The same data as (C), when EJP amplitude in the presence of forskolin, prior to tetanic stimulation, was normalized to 100% (open circles). Comparison with LTF recorded from the same batch of crayfish in the absence of forskolin (closed circles) showed no significant reduction in the amplitude of LTF. The solid line represents recordings of the forskolin response in the absence of tetanic stimulation after normalization.

compared to those experiments performed in the absence of KT5720 (176% ± 21% maintained enhancement), demonstrating that the cAMP-induced maintenance phase is independent of PKA activity. Enhancement is slowly reversible on forskolin wash out (Figures 5A–5C), indicating that cAMP is unable to permanently stabilize synaptic strengthening, in contrast to LTF.

I_h Activation during LTF Temporally "Tags" Synapses

I_h is involved in the induction of both forms of synaptic plasticity, as indicated by the sensitivity of both processes to I_h block. If, as suggested above, I_h is not involved in maintenance of either form of enhancement, then we might be able to replace the I_h requirement in inducing LTF or cAMP-dependent enhancement by prior induction of the alternative enhancement. In this way, I_h activation could be regarded as a temporal synaptic marker or "tag," allowing synapses to respond if given additional appropriate stimuli.

To test this hypothesis, LTF was first induced by delivery of a 20 Hz, 10 min tetanus, prior to application of an I_h blocker, followed by forskolin (30 μM) or 8-Br cAMP (300 μM). Figure 4A showed that this protocol in the absence of I_h blockers resulted in a forskolin or 8-Br cAMP response no different in amplitude from forskolin responses in LTF-naïve preparations. Also, as expected, Figure 6A demonstrates that if this entire protocol was performed with I_h blocked throughout the experiment by ZD7288 (30 μM), neither expression of LTF nor cAMP-dependent enhancement was apparent, with application of forskolin producing only a 12% ± 15% enhancement of EJP amplitude (n = 3). However, Figures 6B–6D show that the cAMP-dependent response developed normally following LTF induction (181% ± 23% enhancement of EJP amplitude with forskolin [n = 6] and 139% ± 19% enhancement of EJP amplitude with 8-Br-cAMP [n = 3]), even when I_h was blocked prior to forskolin application by incubation in either ZD7288 (30 μM) or DK-AH 269 (100 μM). Thus, prior I_h activation during LTF abrogates the need for additional I_h activation by cAMP, and an I_h-independent cAMP response occurs.

As I_h channels are only briefly activated during the LTF-inducing tetanus, this implies that this activation has led to the generation of a more stable "tag," lasting at least 1 hr following its formation. This tag confers

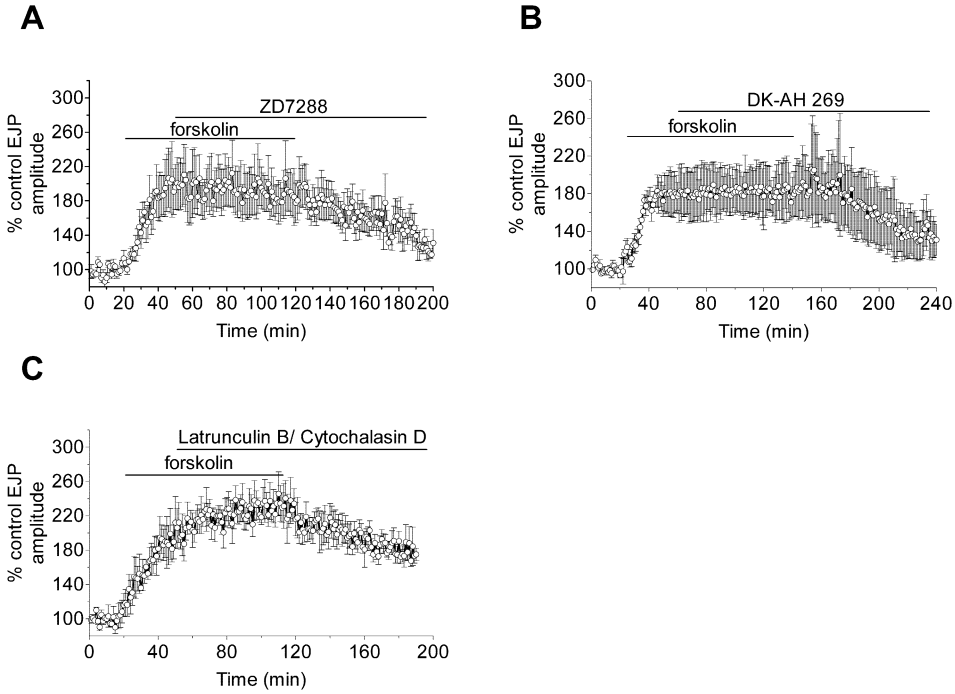

Figure 5. I_h Channel Activation and Intact Actin Cytoskeleton Are Required Only for Induction of cAMP-Dependent Enhancement.

Block of I_h channels by either ZD7288 (30 μM, [A]) or DK-AH 269 (100 μM, [B]) following forskolin (30 μM) failed to disrupt synaptic enhancement, demonstrating that I_h channel activation is critical only for the induction of cAMP-dependent enhancement. (C) Disruption of actin polymerisation by latrunculin B (3 μM) or cytochalsin D (10 μM) applied following incubation with forskolin also fails to affect the subsequent enhancement of transmission, demonstrating that an intact actin cytoskeleton is only required for the I_h-dependent induction of the cAMP response.

onto the synapse the ability to respond to stimuli that would otherwise have required an activation of I_h.

In four of the nine experiments shown in Figures 6B and 6C, the PKA inhibitor KT5720 (1 μM) was included throughout the recording. No difference was seen in either LTF expression (191% ± 20%, KT5720 absent, versus 183% ± 10%, KT5720 present) or the amplitude of cAMP-dependent enhancement (101% ± 10%, KT5720 absent, versus 88% ± 14%, KT5720 present), again confirming the PKA-independent nature of both processes.

I_h Activation by cAMP Fails to Generate a Long-Lasting "Tag"

In Figures 6E and 6F, the same type of experiment was performed in reverse order; that is, cAMP-induced enhancement of transmission was followed by I_h block and subsequent LTF induction. Surprisingly, in this case, a prior cAMP activation of I_h channels did not abrogate the need for I_h activation during LTF induction. There was no subsequent synaptic enhancement during the LTF period following I_h block (2% ± 16%, n = 4), compared with control LTF of 41% ± 9% in the same batch of crayfish in the presence of forskolin alone (see Figure 6F).

One possible explanation for this is that the putative synaptic tag remaining after I_h activation by cAMP lasts only for a limited time, while the synaptic tag generated following LTF induction lasts longer. This possibility, and the mechanism by which this occurs, is examined later.

An Intact Actin Cytoskeleton Is Required for I_h-Dependent Induction of Synaptic Enhancement and Tag Generation

We investigated what role the actin cytoskeleton played in the induction of synaptic enhancement by I_h channel activation and hence tag generation. Figure 7A shows that, as expected, if the LTF protocol and a subsequent forskolin exposure were given following actin depolymerization by either latrunculin B (3 μM) or cytochalasin D (10 μM), both LTF expression and cAMP-dependent enhancement were severely impaired. There was only a 1% ± 9% enhancement of EJP amplitude during LTF, and the subsequent application of forskolin produced a modest 40% ± 19% enhancement of EJP amplitude (n = 4), confirming the importance of the actin cytoskeleton in the induction of both processes (compare with Figure 3D). However, Figure 7B shows that following normal LTF induction, subsequent actin depolymerization by either of the actin depolymerizers did not affect a subsequent cAMP-dependent enhancement, with the enhancement of transmission following forskolin application being 166% ± 23% (n = 3). Thus, a prior LTF induction also abrogates the requirement for an intact actin cytoskeleton to obtain a full cAMP response, implying that actin dynamics are essential to the mechanism of I_h-dependent induction and tag generation and that once the tag is "set," there is no further necessity for actin in the pathway responsible for maintenance of the cAMP response.

When the same experiment was performed in reverse

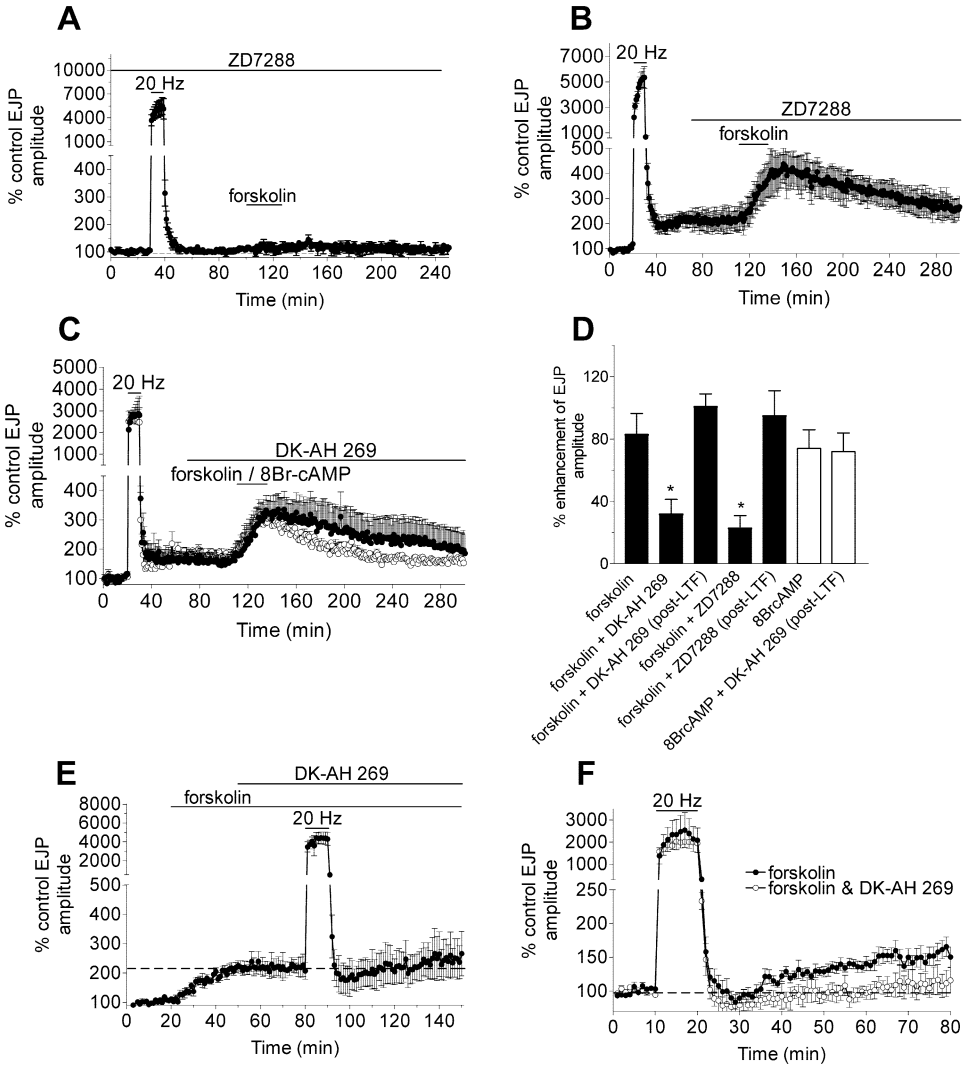

Figure 6. Temporal Synaptic Tagging Mediated by I_h Channel Activation

I_h channel activation during tetanic induction of LTF abrogates the I_h-dependence of a subsequent cAMP-induced enhancement, but not vice-versa.

(A) As expected, block of I_h channels by ZD7288 (30 μM) throughout the experiment results in a failure, both of LTF expression and of a subsequent cAMP-mediated synaptic enhancement.

(B and C) Induction of LTF (and activation of I_h channels) abrogates the requirement for I_h channel activation during subsequent induction of the cAMP response so that the forskolin (closed circles)- or 8-Br cAMP (open circles)-induced enhancement following LTF is no longer sensitive to preapplication of either ZD7288 (B) or DK-AH 269 (C).

(D) Comparing the percent enhancement to forskolin or 8-Br cAMP following LTF induction in the absence of I_h channel block (Figure 4A) and following I_h channel block (B and C) demonstrates that cAMP-dependent enhancement becomes I_h independent following tetanically induced activation of I_h channels. This contrasts with the strong block of cAMP-induced enhancement by preapplication of I_h channel blockers in LTF-naïve preparations (* = $p < 0.05$).

(E) This I_h-dependent "temporal" tagging was not evident in reverse order so that forskolin-induced synaptic enhancement, followed by I_h channel block, did not render subsequent LTF independent of I_h channel activation (closed circles).

(F) LTF was induced in the presence of forskolin (closed circles; see Figure 4C), and in the presence of forskolin followed by I_h channel block (open circles), shown after normalization of EJP amplitude after forskolin application but prior to the tetanus. Under these circumstances, LTF was still prevented in the presence of the I_h channel blocker DK-AH 269.

order, with forskolin application preceding actin depolymerization and subsequent induction of LTF by a 20 Hz, 10 min tetanus (Figure 7C), LTF was still sensitive to actin depolymerization. The tetanus induced an EJP enhancement of only 17% ± 17% in the presence of the actin depolymerizers (n = 5), compared with 176% ± 24% in controls without actin depolymerizers (n = 4). This suggests that either no tag is formed when cAMP is the "conditioning" stimulus or that perhaps only a transient, unstable tag is formed, which is not maintained long enough to abrogate the requirement for actin in the induction of LTF. This is analogous to the continued requirement for I_h in LTF induction following forskolin application described previously (Figures 6E and 6F).

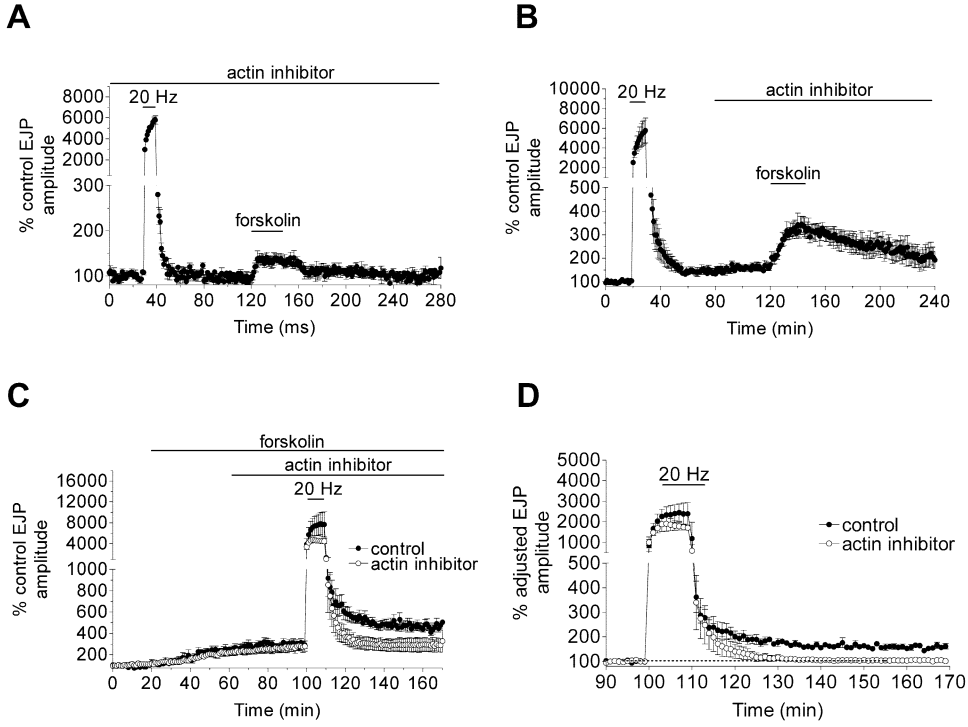

Figure 7. An Intact Actin Cytoskeleton Is Essential for Temporal Synaptic Tagging Mediated by I_h Channel Activation during LTF Induction

Prior I_h-dependent "tagging" during LTF abolishes the actin-dependence of a subsequent cAMP-induced synaptic enhancement, but forskolin-induced enhancement does not abrogate the actin-dependence of subsequent LTF induction.

(A) As expected, disruption of the actin cytoskeleton by the actin depolymerizer latrunculin B (3 μM) prior to LTF induction results in failure, both of LTF expression and of a subsequent full cAMP-mediated synaptic enhancement.

(B) Induction of LTF (and activation of I_h channels) abrogates the requirement for an intact actin cytoskeleton during subsequent induction of the cAMP response so that the forskolin-induced enhancement following LTF is no longer sensitive to preapplication of latrunculin B, indicating that only the I_h-dependent tagging mechanism requires an intact actin cytoskeleton.

(C) This I_h-dependent, actin-dependent "temporal tagging" was not evident in reverse order so that forskolin-induced synaptic enhancement, followed by actin depolymerization, did not render subsequent LTF independent of actin dynamics (open circles), suggesting that no tag is formed following cAMP-mediated enhancement.

(D) LTF was induced in the presence of forskolin (closed circles) and in the presence of forskolin followed by actin depolymerization (open circles), shown after normalization of EJP amplitude after forskolin application but prior to the tetanus. Under these circumstances, LTF was still prevented in the presence of the actin depolymeriser latrunculin B.

I_h- and Actin-Dependent "Temporal Tagging" Occurs Only if I_h Is Activated Coincident with Elevated Presynaptic $[Ca^{2+}]_i$

The occurrence of a long-lasting tag following I_h activation by LTF induction, but not by cAMP, was investigated. We have previously shown that in addition to I_h activation, LTF induction also relies on elevation of presynaptic $[Ca^{2+}]_i$, and LTF expression is critically dependent on activation of calcineurin and a number of protein kinase pathways and on presynaptic protein synthesis (Beaumont et al., 2001). In contrast, none of these pathways is required for cAMP-dependent enhancement. We therefore examined whether activation of any of these process involved specifically in LTF are also involved in conferring a long-lasting temporal I_h- and actin-dependent tag.

First, we investigated whether protein synthesis is required to stabilize the I_h- and actin-dependent tag formed following LTF induction. Expression of LTF depends on de novo presynaptic protein translation (Beaumont et al., 2001), and LTF expression is abolished in the presence of the translation inhibitor anisomycin (20 μM, n = 3; Figure 8A). However, block of protein synthesis did not affect tag formation—a subsequent cAMP response remained unaffected by preapplication of the I_h blocker ZD7288, with the percent enhancement of EJP amplitude in response to forskolin being 112% ± 27%. Thus, persistent tag formation occurs independently of protein synthesis or LTF induction.

We next investigated the potential contribution of the multiple protein phosphatase/kinase pathways that are activated and involved in LTF generation in the maintenance of the tag. As expected, a cocktail of inhibitors of calcineurin, MAP kinase, phosphatidylinositol-3-kinase, and mTOR/FRAP prevented LTF expression (3% ± 4% enhancement of EJP amplitude). But this cocktail left intact the I_h- and actin-dependent tag formed during LTF expression—a subsequent forskolin response was still I_h independent, resulting in a 113% ± 17% enhancement of EJP amplitude (n = 6) in the presence of ZD7288 (30 μM; Figure 8B). Thus, none of these enzymes contributes to persistent tag formation.

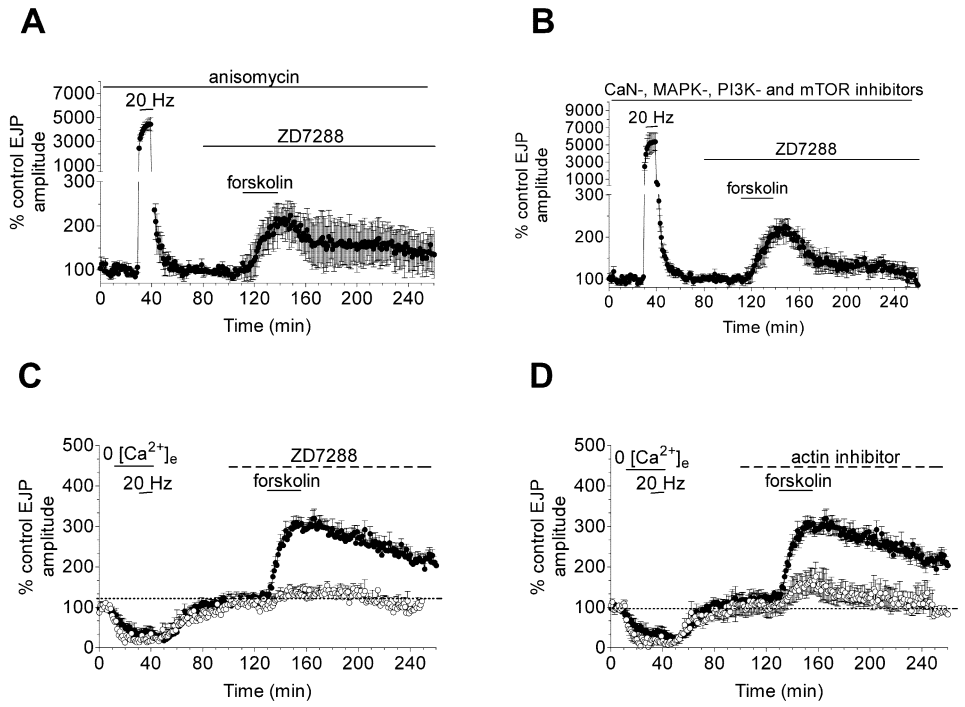

Figure 8. I_h- and Actin-Dependent Temporal Tagging Occur Only if I_h channels Are Activated Coincident with Elevated Presynaptic Calcium

(A) The abrogation of I_h dependence of the cAMP response, and hence tag generation, were not disrupted when protein synthesis, and consequently LTF expression, were inhibited by application of anisomycin (20 μM).

(B) Similarly, inhibition of the phosphatases and kinases implicated in LTF-calcineurin, MAPkinase, PI3 kinase, and mTOR/FRAP by a cocktail of inhibitors (ascomycin [300 nM], cyclosporine A [30 μM], PD098059 [30 μM], wortmannin [100 nM], and rapamycin [100 nM]) prevents LTF expression but fails to disrupt the I_h-dependent tag.

(C and D) In contrast, if LTF expression is prevented by delivering the LTF-inducing tetanus in a calcium-free external media (0 $[Ca^{2+}]_e$), no tagging occurs, and a subsequent forskolin response remains susceptible to both I_h channel block using ZD7288 (30 μM, [C]; open circles) and actin disruption by latrunculin B (3 μM, [D]; open circles), as compared to the forskolin responses in the absence of these inhibitors following LTF induction in 0 $[Ca^{2+}]_e$ (C and D; closed circles).

LTF induction also requires presynaptic Ca^{2+} accumulation during an LTF-inducing tetanus, as subsequent LTF is prevented if the tetanus is delivered in a Ca^{2+}-free medium (Beaumont et al., 2001). We have therefore tested whether elevation in presynaptic $[Ca^{2+}]_i$ is required to stabilize the I_h- and actin-dependent tag. The LTF tetanus was delivered after exchange of the external medium with one containing no Ca^{2+} and 0.75 mM EGTA, and then Ca^{2+} was reintroduced (Figures 8C and 8D). Under these conditions, when EJP amplitude recovered, LTF was prevented, and subsequently a normal cAMP-dependent response was obtained, showing a 169% ± 16% enhancement (n = 5). However, when either ZD7288 (30 μM; Figure 8C) or the actin depolymerizers latrunculin B (3 μM) or cytochalasin D (10 μM) was applied after the LTF-inducing tetanus, but prior to forskolin, the subsequent cAMP enhancement was blocked (29% ± 15% and 53% ± 16% enhancement respectively, n = 3 each; Figures 8C and 8D). This suggests that without concomitant elevation in presynaptic $[Ca^{2+}]_i$, tag generation is either prevented or it is not sufficiently stable to be maintained long enough to render the cAMP response I_h and actin independent. Thus, concomitant $[Ca^{2+}]_i$ elevation, or some process dependent upon Ca^{2+} influx, appears to be essential for persistent temporal tag formation.

Discussion

Our results reveal a novel mechanism for the induction of long-term synaptic plasticity, relying on accumulation of presynaptic Na^+ and activation of the Na^+/K^+ ATPase during extensive high-frequency activity, with resultant presynaptic hyperpolarization and activation of I_h. Our data suggest that I_h activation is critical for the induction of both LTF and the short-term enhancement mediated by cAMP and that this process depends on an intact actin cytoskeleton. However, the induction and expression of LTF also require presynaptic Ca^{2+} influx (or some process dependent on Ca^{2+} influx) and local protein translation, and activation of the Ca^{2+}-dependent phosphatase calcineurin, and also of MAP, PI3, and mTOR/FRAP kinases (Beaumont et al., 2001). cAMP-dependent enhancement does not utilize the complex pathways involved in maintenance of LTF, and thus LTF- and cAMP-dependent enhancement are nonocclusive. While cAMP-dependent enhancement also involves I_h-activated synapses, enhancement is maintained only for as long as the cyclase is activated, and presumably as long as cAMP is present. Moreover, we have the finding of Beaumont and Zucker (2000) that PKA is not involved in any aspect of the cAMP response. cAMP-dependent, PKA-independent synaptic strengthening has been de-

scribed at the Calyx of Held synapse (Sakaba and Neher, 2001), where cAMP increases the Ca^{2+} sensitivity for secretion, apparently by selectively increasing the number of docked vesicles that are sensitive to Ca^{2+}. The cAMP binding sites are undetermined, but one candidate is a recently identified cAMP-sensitive guanine nucleotide exchange factor involved in Ras signaling (de Rooij et al., 1998; Kawasaki et al., 1998).

How does I_h activation influence synaptic strength? We hypothesize that I_h activation initiates a process leading to a "marking" of synapses, allowing them to respond within the lifetime of this marker to additional stimuli to which they would otherwise be refractory. This idea may be related to the spatial synaptic "tagging" proposed by Frey and Morris (1997) to explain certain aspects of the input specificity of mammalian cortical long-term potentiation (LTP). They suggested that LTP initiated the creation of a short-lasting postsynaptic protein synthesis-independent "synaptic tag" at the potentiated synapse, allowing the subsequent selective sequestration of relevant proteins to establish late LTP. In support of this idea, they showed that weak tetanic stimulation, which ordinarily leads only to an early LTP, or repeated tetanization in the presence of protein synthesis inhibitors, could result in late LTP, provided that repeated tetanization had already been applied at another input to the same population of neurons. In contrast to this spatial tagging in LTP, at crayfish neuromuscular junctions, a form of temporal synaptic tagging occurs presynaptically.

If I_h activation initiates events leading to a synaptic tag, then prior I_h activation by either cAMP or by hyperpolarization occurring during tetanic stimulation, followed immediately by I_h block, should result in a transient "tag." This would allow a response to the processes responsible for maintenance of the alternate form of synaptic enhancement, provided that the alternate stimulus was delivered within a temporal window defined by the lifetime of the I_h- and actin-induced tag.

Supporting this idea, we found that I_h activation induced by tetanic stimulation could abrogate the requirement for I_h activation and full actin integrity in a subsequent cAMP-dependent enhancement, suggesting that presynaptic I_h activation can "prime" synapses to respond more robustly to subsequent stimuli.

However, in contrast, cAMP-dependent enhancement does not result in an abrogation of the requirement for I_h activation and an intact actin cytoskeleton in the subsequent induction of LTF. This led to an extension of the previous hypothesis, namely that activation of I_h could generate a short-lived tag (requiring that the actin cytoskeleton remain intact)—but that additional processes occurring during the induction of LTF are necessary to stabilize this "tag." Our results suggest that the presynaptic $[Ca^{2+}]_i$ elevation occurring during an LTF-inducing tetanus is a key event leading to tag stabilization. As presynaptic $[Ca^{2+}]_i$ elevation does not occur during a cAMP stimulus, the "tag" formed in response to the subsequent activation of I_h is transient and unable to render subsequent LTF I_h- and actin-independent.

What is the structural nature of the I_h-dependent tag? Our data indicate that both induction of cAMP-dependent enhancement and the tag generated following LTF are susceptible to agents that disrupt actin polymerization, suggesting that the integrity of the actin cytoskeleton is an important component of I_h-dependent tagging. One simple explanation is that actin stabilizes the localization of I_h channels at synapses, or sustains interactions between I_h channels and secretory proteins, perhaps in the same way that actin stabilizes clusters of K^+ channels in central neurons (Petrecca et al., 2000). However, we cannot discount the possibility that actin may play a more dynamic role in the generation of I_h-dependent synaptic tags. Whatever the case, given the relatively transient activation of the ion channel leading to tag formation, it appears that the tag generated occurs as a result *of* I_h activation and is not the I_h channel itself.

That I_h activation can tag synapses is intriguing, and it will be interesting to discover to what extent similar mechanisms are involved in regulation of vertebrate central synapses. The generation of a robust Na^+/K^+ ATPase exchange current in response to a tetanus concomitant with presynaptic $[Ca^{2+}]_i$ elevation allows I_h channels to act as detectors of sustained high-frequency activity, tagging those synapses that are most strongly activated. What might be the functional role of "temporal synaptic tagging" by LTF? Tagging renders the cAMP-dependent enhancement of synaptic transmission I_h independent. cAMP activation of I_h channels is voltage dependent, and moderate depolarization blocks the activation of these channels by cAMP (Wainger et al., 2001). Thus, tetanic tagging of synapses might protect them from a subsequent depolarization-induced block of enhancement to cAMP-dependent extrinsic signals.

Experimental Procedures

Dissection and Electrophysiological Recording

The crayfish opener muscle dissection (Landò and Zucker, 1994) and intracellular electrophysiological recording apparatus have been described in detail elsewhere (Beaumont et al., 2001; Beaumont and Zucker, 2000).

Fluorescence Imaging of Presynaptic $[Na^+]_i$

The exciter axon was penetrated with a beveled microelectrode (25–45 MΩ) containing 20 mM SBFI in 200 mM KCl, and 10 mM HEPES, pH 8.5; dye was iontophoresed (−10 nA, ~30 min) to a final SBFI concentration of ~0.5 mM. $[Na^+]_i$ was measured as previously described for fura-2 measurement of $[Ca^{2+}]_i$ (Mulkey and Zucker, 1992), with the exception that a neutral density filter was used to reduce excitation light intensity by half to minimize photobleaching.

SBFI was calibrated in situ (Harootunian et al., 1989). Axons injected with SBFI were permeabilized by addition of Na^+ ionophores palytoxin (0.1 μM, which also blocks Na^+/K^+ exchange), gramicidin D (10 μM), and monensin (10 μM), and subsequently perfused with solutions comprising varying $[Na^+]$. This was achieved by mixing a "high Na^+" solution comprising 13 mM NaCl, 244 mM Na-gluconate, 10 mM Cs-HEPES, 5 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.00, with a "high K^+" solution containing 13 mM KCl, 244 mM K-gluconate, 10 mM Cs-HEPES, 5 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.00, so that ionic strength remained at 0.28 Osm and $[Na^+]$ could be set from 0 to 250 mM. Ratiometric fluorescence images were obtained at different $[Na^+]$. R_{max}/R_{min} was 1.88 and the K_d of Na^+ binding to SBFI was determined as 21 ± 2 mM (n = 3) using Equation 5 of Grynkiewicz et al. (1985).

Drugs

ZD7288 was obtained from Tocris Cookson (Ballwin, MO). DK-AH 269 was from Boehringer Ingelheim (Germany). The actin depolymerizers cytochalasin D, latrunculin B, and swinholide A were obtained from Biomol (Plymouth Meeting, PA). 8-Br-cAMP was from

Sigma (St Louis, MO). All other drugs were from Calbiochem (La Jolla, CA). In some cases (forskolin, latrunculin B, ascomycin, anisomycin, wortmannin, and rapamycin), stock solutions of drugs were prepared in DMSO and dissolved before use in external media to a final concentration containing not more than 0.1% DMSO. Control experiments using 0.1% DMSO showed that LTF expression and synaptic tagging following LTF induction were not affected by addition of this solvent ($n = 2$ each).

Presynaptic Injection Procedures

Pressure injection of the impermeable compound SQ 23566 (16 mM) in a dye-marked carrier solution (0.5 mM fluorescein, 200 mM KCl, and 10 mM HEPES, pH 7.4) was performed by positive pressure pulses (30–40 psi, 1 s) applied to a microelectrode lumen until fluorescein was detected (at 480 nm illumination) throughout the axonal arbor (normally after ~30 min). The intensity of fluorescence in the axon was used to estimate the intracellular concentration of fluorescein and by extrapolation that of SQ 23566, according to methods previously described (Delaney et al., 1989).

Statistics

Statistical analysis of results was performed using Student's unpaired t test and significance assumed at $p < 0.05$. When LTF in the presence of drugs was compared to control LTF, either graphically or quantitatively in the text, this was done against controls obtained from the same batch of crayfish to avoid erroneous interpretation of drug effects that may have occurred due to seasonal, batch, or other variations.

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