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Direct Modulation of the Secretory Machinery Underlies PKA-Dependent Synaptic Facilitation in Hippocampal Neurons

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Summary

Activation of protein kinase A (PKA) is known to facilitate synaptic transmission. Using synapses established by hippocampal neurons in culture, we show that dialysis of PKA inhibitors in the presynaptic neuron blocks synaptic facilitation produced by the adenylyl cyclase activator forskolin, demonstrating a presynaptic locus of action. Using ruthenium red, a tool that is known to stimulate exocytosis independently of Ca^{2+} influx, but in a manner sensitive to tetanus toxin, we find that the secretory process is directly up-regulated under conditions where the number of functional terminals remains unchanged, as revealed by imaging of FM1-43, a vital indicator of synaptic vesicle endocytosis. Taken together with our ultrastructural analysis that suggests no enhancement of docking, our data indicate that PKA causes synaptic facilitation by directly elevating the probability of exocytosis of individual vesicles in response to an invariant Ca^{2+} signal.

Introduction

A large number of proteins present on synaptic vesicles and on the presynaptic membrane of nerve terminals have been identified and cloned in the past few years (Südhof, 1995; Scheller, 1995). Some of these can be phosphorylated by various protein kinases (Greengard et al., 1993; Fykse et al., 1995; Nielander et al., 1995). This raises the possibility that the secretory process in neurons may be directly modulated by phosphorylation, as suggested by previous work in invertebrates (Man-Son-Hing et al., 1989). This type of regulation of the secretory process by phosphorylation may be a general process because in endocrine cells, a similar facilitation or "priming" of exocytosis may depend on Ca^{2+} (Neher and Zucker, 1993; von Rüden and Neher, 1993), but also on Mg–ATP, the latter effect being blocked by protein kinase inhibitors (Bittner and Holz, 1992; Hay and Martin, 1992, 1995; Parsons et al., 1995).

In many preparations, activation of the cAMP–protein kinase A (PKA) pathway has been shown to facilitate synaptic transmission (Dixon and Atwood, 1989; Chavez-Noriega and Stevens, 1994; Klein, 1994; Weisskopf et al., 1994; Capogna et al., 1995), apparently by a presynaptic mechanism. The finding that the frequency but not the amplitude of spontaneous miniature synaptic events can be enhanced by forskolin also supports a presynaptic mechanism (Capogna et al., 1995). One possibility is that the facilitation may be caused by an enhancement of resting Ca^{2+} or Ca^{2+} influx through voltage-gated channels. Alternatively, the secretory process may be directly facilitated, thereby allowing an increase in the number of vesicles released for any given Ca^{2+} signal. We have tested these models at terminals established by hippocampal neurons in culture. We find that presynaptic activation of PKA is necessary for the facilitation produced by the adenylyl cyclase activator forskolin, confirming a presynaptic locus of change. Furthermore, the facilitation, expressed under conditions where changes in Ca^{2+} dynamics are undetectable, is accompanied by an enhancement of the secretory response to ruthenium red (RR), a Ca^{2+}-independent secretagogue. Finally, because the number of functional terminals and of morphologically docked vesicles is unchanged, we suggest that presynaptic activation of PKA directly facilitates the secretory process at a step downstream from Ca^{2+} influx.

Results

Dual Whole-Cell Recordings of Evoked Synaptic Currents

Most experiments were performed at synapses established in culture by rat hippocampal interneurons releasing the neurotransmitter γ-aminobutyric acid (GABA). Under our culture conditions, approximately two thirds of cell pairs tested are connected by GABAergic synaptic contacts (data not shown). Activation of adenylyl cyclase by the membrane-permeant agent forskolin facilitates inhibitory postsynaptic currents (IPSCs) in the hippocampus (Capogna et al., 1995). In dual whole-cell patch-clamp recording experiments, forskolin (20 μM) produced more than a 2-fold increase in the amplitude of monosynaptic IPSCs evoked by a presynaptic action potential ($+126.2\% \pm 18.5\%$, $n = 7$) (Figures 1A and 2A). This facilitation was reversible within 2 min of washout (Figure 1B). To determine whether the predominant site of modulation was pre- or postsynaptic, protein kinase inhibitors were included in the pre- and/or postsynaptic patch pipettes. Simultaneous dialysis of the serine/threonine kinase inhibitor H7, both pre- and postsynaptically, completely blocked the forskolin-induced synaptic facilitation ($+6.3\% \pm 2.7\%$, $n = 5$, $p < 0.01$, Fisher post hoc test; one-way ANOVA $F(6,38) = 10.8$, $p < 0.01$ overall for groups shown in Figure 2A). Presynaptic dialysis of H7 similarly blocked facilitation ($+19.0\% \pm 10.7\%$, $n = 5$, $p < 0.01$), while postsynaptic dialysis had no statistically significant effect on the facilitation ($+66.0\% \pm 25.7\%$, $n = 5$, not significant). The trend toward a small reduction of facilitation in the latter case may result from diffusion of H7 across the synaptic cleft due to the membrane permeability of this drug. Specific inhibitors were used to test the presynaptic involvement of PKA further. Inclusion of either Rp-cAMPS (50 μM) or a peptide inhibitor of PKA, PKI(5–24) (50 μM), in the presynaptic pipette significantly reduced the synaptic facilitation induced by forskolin ($+8.3\% \pm 5.7\%$, $n = 6$, $p < 0.01$; and $+33.6\% \pm 11.1\%$, $n = 6$, $p < 0.01$; respectively) (Figure 2A). Finally, direct activation of PKA by

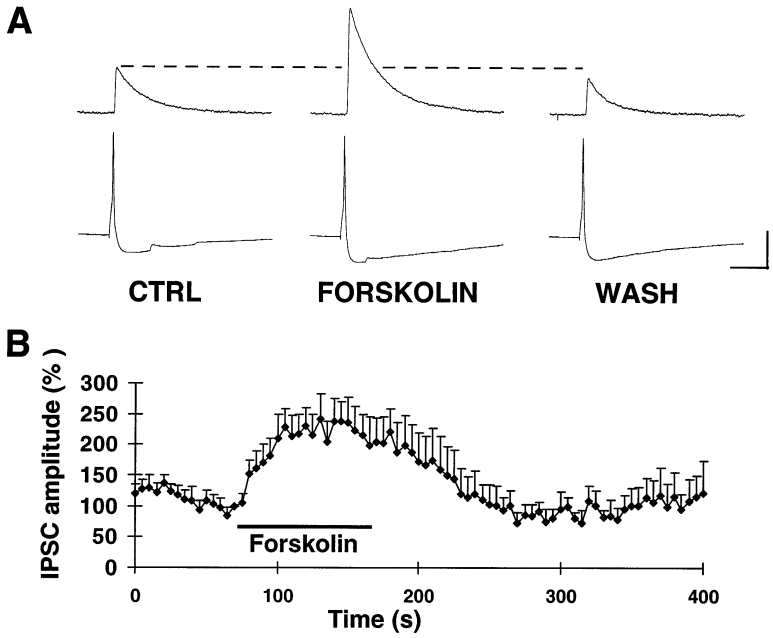

Figure 1. Facilitation of Action Potential–Evoked IPSCs by Forskolin

(A) Dual whole-cell patch-clamp recordings obtained from a pair of rat hippocampal neurons in primary culture. An action potential evoked in the unclamped presynaptic neuron (bottom traces) evoked an IPSC in the postsynaptic neuron voltage clamped at −40 mV (top traces). Perfusion of 20 μM forskolin to promote cAMP production in the neurons enhances the size of the IPSC. Calibration: 8 nS, 35 mV, 40 ms.

(B) Time course of forskolin-induced synaptic facilitation. The effect of forskolin was reversible upon washout. The presynaptic neuron was stimulated to produce an action potential at 0.2 Hz. Forskolin was applied for approximately 100 s (bar) following an initial control period and was then washed out with normal saline. The amplitude of the IPSCs (mean ± SEM) are plotted relative to the last control IPSC of each experiment.

intracellular dialysis of the nonhydrolyzable cAMP analog Sp-cAMPS (1 mM) in the presynaptic neuron also produced facilitation of evoked IPSCs, an effect that developed over a 2 min period (Figure 2B) (n = 5). Taken together, these results demonstrate the involvement of presynaptic PKA in the facilitation produced by forskolin at the synapse.

Measurements of Intracellular Ca^{2+} Levels

Ion imaging experiments have demonstrated that a sustained elevation in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in central neurons can enhance both action potential–evoked synaptic currents as well as spontaneous miniature synaptic currents (McGehee et al., 1995). To determine whether a similar mechanism could explain the synaptic facilitation induced by forskolin, we monitored $[Ca^{2+}]_i$ by quantitative ratiometric imaging of fura-2. We detected no effect of forskolin (20 μM) on resting $[Ca^{2+}]_i$ or on depolarization-evoked rises in Ca^{2+} in the cell bodies and neurites of neurons (Figure 2C). Resting $[Ca^{2+}]_i$ changed by an average of -4 ± 3 nM upon application of forskolin (20 μM), while the depolarization-evoked elevation in $[Ca^{2+}]_i$ was 344 ± 79 nM before forskolin and 290 ± 61 nM after (paired t test, not significant, n = 6). Although highly localized changes in Ca^{2+} handling might have gone undetected in these experiments, our results suggest that the presynaptic facilitation produced by forskolin does not result from a change in $[Ca^{2+}]_i$ dynamics.

Ca^{2+}-Independent Secretory Response

To determine whether activation of the cAMP–PKA pathway could directly enhance the efficacy of the secretory process at a point beyond Ca^{2+} entry into the nerve terminal, we took advantage of an approach that we have recently developed in which vesicle exocytosis is triggered in the absence of either Ca^{2+} influx or elevation of resting Ca^{2+} levels. Brief application of the polyvalent cation RR to nerve terminals produces a rapid (<1 s onset), reversible, and reproducible elevation of the frequency of miniature synaptic currents in a Ca^{2+}-independent manner, by binding to external sites on the presynaptic membrane (Trudeau et al., 1996). Like Ca^{2+}-evoked neurotransmitter release, this secretory response is tetanus toxin (TeTx) sensitive (Figure 3). Dual whole-cell patch-clamp recordings were obtained from pairs of cultured hippocampal neurons. In control cultures, action potential–evoked postsynaptic currents were detected in 16 out of 17 (94%) tested cell pairs (Figure 3A). In parallel cultures treated overnight with 33 nM TeTx to cleave the synaptic protein synaptobrevin (Schiavo et al., 1992), 0 of 22 cell pairs tested showed action potential–evoked synaptic transmission (Figure 3A). The treatment was therefore effective at blocking Ca^{2+}-dependent neurotransmitter release. TeTx was also found to block RR-evoked neurotransmitter release. In the presence of tetrodotoxin (TTX) to block action potentials, whole-cell recordings were obtained from neurons, while a second pipette was brought in close proximity to pressure-eject RR onto synaptic terminals within the field of view. In these experiments, RR-evoked release was measured as the difference between the number of miniature synaptic currents (minis) detected before and after RR, within 15 s recording periods. In control cultures, RR always evoked a brisk increase in the frequency of occurrence of minis (Figure 3B). In TeTx-treated cultures, the effect of RR on secretion was completely blocked ($p < 0.001$). This was observed both for SR-95531-sensitive GABAergic inhibitory events (mIPSCs) and for CNQX-sensitive glutamatergic excitatory events (mEPSCs) (Figure 3C). These results show that secretion induced in a Ca^{2+}-independent manner by RR is dependent upon the same synaptic protein complex that is required for Ca^{2+}-dependent secretion (Scheller, 1995).

Here, we used RR-evoked release as an assay to test

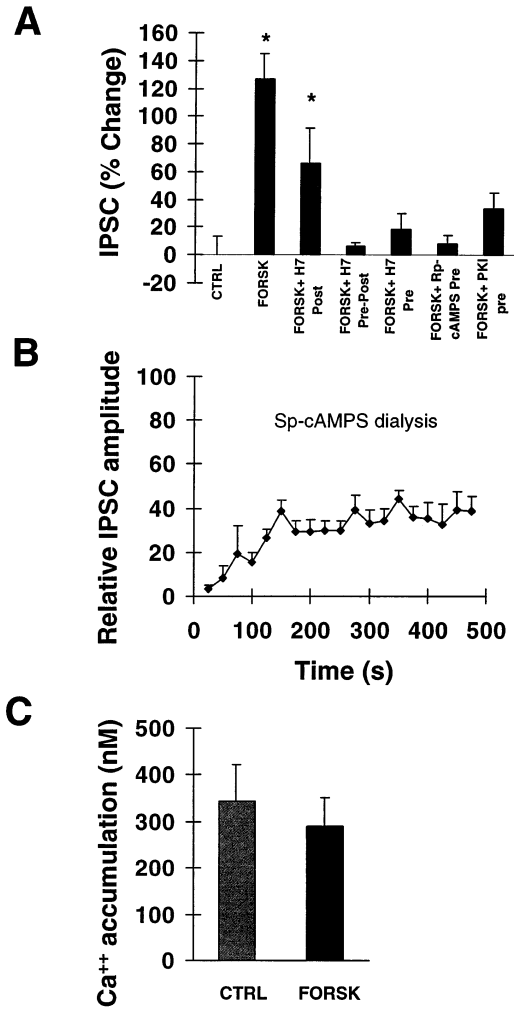

Figure 2. PKA-Dependent Presynaptic Facilitation

(A) Summary bar graph of the synaptic facilitation experiments. Significant facilitation, as compared with vehicle alone (CTRL), was obtained with 20 μM forskolin by itself (FORSK), or when the protein kinase inhibitor H7 (50 μM) was included in the postsynaptic patch pipette (H7 Post). No significant facilitation was obtained when H7 was included both pre- and postsynaptically (H7 Pre-Post) or presynaptically alone (H7 Pre), or when the PKA antagonists Rp-cAMPS (50 μM) or the peptide PKI(5–24) (50 μM; Sigma) were included presynaptically. Asterisk indicates $p < 0.05$.

(B) Time course of synaptic facilitation (%) produced by intracellular dialysis of the PKA-activator Sp-cAMPS in presynaptic neurons ($n = 5$). The IPSCs were evoked every 5 s and their amplitude expressed relative to the average of the first five IPSCs of each experiment. Every fifth consecutive IPSC was averaged and normalized to the average IPSC amplitude of parallel control experiments (within each bin), without Sp-cAMPS in the pipette, to account for the gradual rundown of synaptic currents observed in control experiments.

(C) In other cells, the elevation in Ca^{2+} produced by depolarizing neurons with saline containing 22 mM K^+ was not found to be significantly changed by treatment with forskolin. The synaptic facilitation produced by forskolin also was not found to be accompanied by any detectable change in resting $[Ca^{2+}]_i$ levels (data in text).

whether the secretory process can be directly facilitated upon exposure of neurons to forskolin. Miniature inhibitory synaptic currents (mIPSCs) were recorded in the presence of TTX for periods of 15 s before and after puff application of 30 μM RR. The secretory response (number of mIPSCs) was again computed as a difference score and was evaluated before and after 3 min of perfusion with 20 μM forskolin. The secretory response to RR was dramatically enhanced following the addition of forskolin (+197% ± 41%, $n = 8$) (Figures 4A and 4B), in comparison to control preparations exposed to the vehicle alone (0.1% DMSO, +35% ± 21%, $n = 4$, $p < 0.05$, Fisher post hoc test; one-way ANOVA $F(3,24) = 3.5$, $p < 0.05$ overall, for groups shown in Figure 4B). Preexposure of the neurons to 50 μM Rp-cAMPS, a PKA antagonist, attenuated the facilitory action of forskolin (+53% ± 25%, $n = 5$; significantly different than with forskolin alone, $p < 0.05$) (Figure 4B). To confirm further that the pathway activated by forskolin acts on the secretory apparatus per se rather than through Ca^{2+} influx, we repeated the experiments in the presence of 1 mM external $CoCl_2$, a broad-spectrum Ca^{2+} channel blocker. As expected if the facilitation of neurotransmitter release was downstream from the Ca^{2+} signal, we found that the forskolin-induced facilitation of RR-evoked release was unhampered in the presence of $CoCl_2$ (+282% ± 93%, $n = 5$) (Figure 4B).

In accordance with the strictly presynaptic nature of the facilitation produced by forskolin, we found that the amplitude distribution of mIPSCs evoked by RR was unchanged by forskolin (Figure 4C) ($n = 6$; $F(1,487) = 2.32$, $p > 0.05$ [for CTRL versus FORSK main effect], two-way ANOVA). Additionally, currents evoked by the direct application of GABA to the neurons (sensitive to the $GABA_A$ antagonist SR-95531) were unchanged by forskolin (Figure 4D) (−3% ± 3% change, $n = 5$, paired t test, $p > 0.05$).

Detection of Active Synaptic Terminals with FM1-43

Facilitation of the secretory process could occur through a number of presynaptic mechanisms. For example, some synaptic terminals may initially be silent but gain the ability to release neurotransmitter following PKA-dependent phosphorylation. We tested this hypothesis by identifying active presynaptic terminals using the fluorescent label of recycling synaptic vesicles FM1-43. This styryl dye is sequestered by synaptic vesicles during endocytosis (Betz and Bewick, 1992; Ryan et al., 1993; Henkel at al., 1996a). Its uptake in synaptic terminals is activity dependent: it requires Ca^{2+} influx and is blocked by clostridial neurotoxins (Henkel et al., 1996b). Synaptic terminals were loaded with FM1-43 using a depolarizing stimulus (40 mM external K^+, 60 s) and then, after 10 min, were depolarized again with the same stimulus to induce exocytosis and destaining. Images acquired before and after destaining were digitally subtracted to identify active synaptic terminals. These appeared as fluorescent "puncta," which were counted blind. This protocol was performed twice, both before and after application of 20 μM forskolin for 3 min. We found no evidence supporting the systematic appearance of new functional synaptic terminals (Figure 5A). In preparations exposed to forskolin, the average number of fluorescent puncta increased by 8% ± 10% ($n = 6$) from the first to the second FM1-43 loading protocol, in comparison to a 14% ± 16% ($n = 5$) increase in control

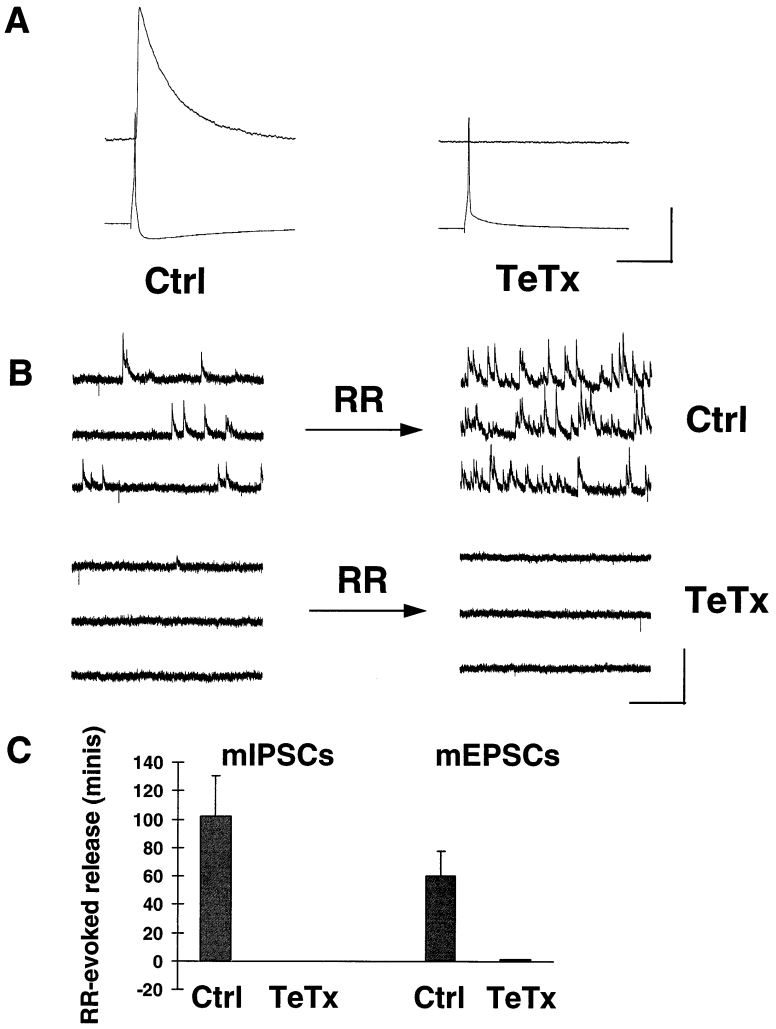

Figure 3. Activation of the Secretory Process by RR Is Blocked by TeTx

(A) Dual whole-cell recordings from hippocampal neurons in culture. In control cultures (left), a presynaptic action potential in one of the neurons (lower trace) usually induced a postsynaptic current in the other (top trace). In this example, an IPSC was recorded from a holding potential of −20 mV. Of 17 control cell pairs tested, 16 were synaptically connected, 6 displayed EPSCs, and 10 displayed IPSCs. In cultures treated with TeTx, action potentials were unimpaired, but they failed to induce a detectable postsynaptic response (top trace). Calibration: 400 pA, 50 mV, 55 ms.

(B) Whole-cell recordings from neurons at a holding potential of 0 mV in the presence of CNQX and TTX. In control cultures (top set of traces), the frequency of occurrence of mIPSCs is briskly increased by puff application of RR (30 μM) (arrow). In cultures treated with TeTx (lower set of traces), RR fails to induce any change in mIPSC frequency. Calibration: 42 pA, 575 ms.

(C) Summary. RR-evoked release is presented as a difference score between the number of minis recorded in a 15 s period before and after puff application of RR. The induction of both mIPSCs (left bars) and mEPSCs (right bars) is blocked by preincubation with TeTx. For control experiments, n = 4 for mIPSCs and n = 10 for mEPSCs. For TeTx, n = 8 for mIPSCs and n = 13 for mEPSCs.

preparations (t test, $p > 0.05$). These results suggest that forskolin did not activate previously "silent" terminals. They are compatible with a model of facilitation that involves an enhancement of the secretory process at previously functional synaptic terminals.

Forskolin Does Not Modify Synaptic Ultrastructure

Two major mechanisms of secretory apparatus modulation could lead to an enhancement of transmitter release from functional synapses: either the number of vesicles docked at active zones of the presynaptic membrane in preparation for exocytosis is enhanced approximately 2-fold, or alternatively, the probability of vesicle exocytosis is augmented with no change in the proportion of docked vesicles. To distinguish between these possibilities, we investigated quantitatively the ultrastructure of nerve terminals (Figure 5B). We could find no significant increase in the number or percentage of vesicles docked to the presynaptic membrane (defined here as within 50 nm of the presynaptic membrane). In sections from forskolin-treated terminals, 10.1% ± 1.4% of vesicles were docked on average (7.5 ± 1.3 vesicles/section, n = 16), in comparison to 10.9% ± 1.2% for control terminals (6.9 ± 0.7 vesicles/section, n = 14). To prevent any effect of forskolin on ultrastructure being masked by an increase in spontaneous exocytosis, parallel cultures were also pretreated overnight with TeTx to cleave the synaptic vesicle protein synaptobrevin and block exocytosis. Comparing cultures treated with TeTx alone and TeTx followed by forskolin, we again found no evidence for any increase in vesicle docking. In sections from forskolin-treated terminals, 9.8% ± 1.9% of vesicles were docked on average (5.8% ± 1.1 vesicles/section, n = 13), in comparison to 10.1% ± 1.4% for control terminals (8.2 ± 0.9 vesicles/section, n = 14). These data are compatible with the idea that vesicle docking is not enhanced, indicating that forskolin acts mainly by modifying the probablility of vesicle exocytosis.

Discussion

Our observations present evidence for a direct modulatory control of the secretory machinery of synaptic terminals by a second messenger system. The facilitation of synaptic transmission produced by the adenylyl cyclase activator forskolin could result from the presynaptic production of cAMP leading to the activation of PKA, which could then phosphorylate one or more proteins, either

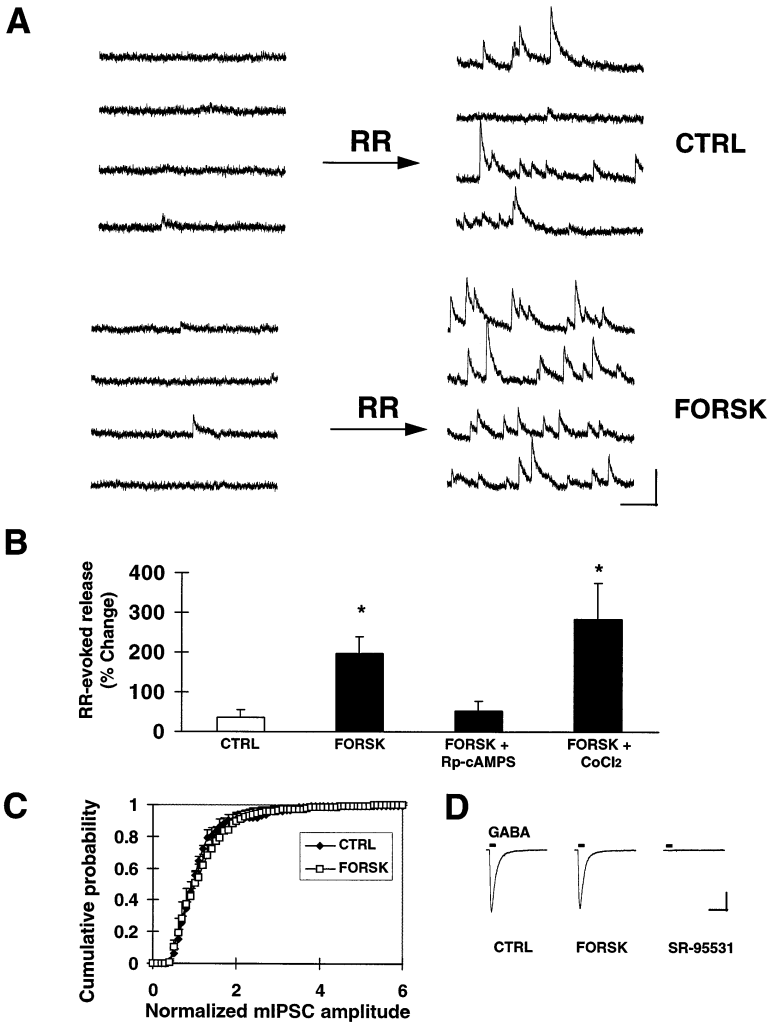

Figure 4. Forskolin Facilitates GABAergic Synaptic Transmission by Direct Modulation of the Secretory Process

(A) mIPSCs recorded in hippocampal neurons in the presence of TTX and 10 μM CNQX at a postsynaptic holding potential of 0 mV. Puff application of 30 μM RR to the neurons produced a brisk elevation in the frequency of occurrence of the mIPSCs (top set of traces). Perfusion of 20 μM forskolin (FORSK) for 3 min produced an enhancement of the secretory response to a subsequent puff application of RR (bottom set of traces). Calibration: 1.1 nS, 180 ms.

(B) Summary histogram. Forskolin produced a significant increase in the RR-evoked secretory response. The effect was blocked by preincubation for >5 min with the PKA antagonist Rp-cAMPS (50 μM) (FORSK + Rp-cAMPS), but not by blocking Ca^{2+} channels with 1 mM $CoCl_2$ (Forsk + $CoCl_2$). Asterisk indicates $p < 0.05$.

(C) Cumulative probability distribution of the amplitude of mIPSCs evoked by RR before (CTRL) and after forskolin (FORSK). The two distributions were not significantly different.

(D) Whole-cell currents, produced at a holding potential of −80 mV, by the direct application of 500 μM GABA from a nearby pipette by brief (20 ms) pressure ejection. These currents were unaffected by 20 μM forskolin (FORSK; middle trace), but were blocked by the $GABA_A$ receptor antagonist SR-95531 (10 μM; right trace). Calibration: 4.5 nS, 550 ms.

associated with, or part of, the protein complex that is necessary for the exocytosis of synaptic vesicles (Südhof, 1995; Scheller, 1995). This type of mechanism could be implicated in the control of neurotransmitter release by a wide variety of presynaptic receptors present in the central nervous system.

The finding that restings levels of Ca^{2+} and depolarization-evoked rises in $[Ca^{2+}]_i$ are not affected by forskolin is compatible with a facilitation of the secretory process downstream from Ca^{2+} influx or accumulation in synaptic terminals. The mechanism would therefore differ from that responsible for presynaptic facilitation produced by nicotine at central synapses (McGehee et al., 1995). However, because we have not performed single terminal measurements of Ca^{2+} levels in response to action potentials, it remains possible that small changes in Ca^{2+} dynamics restricted to nerve terminals would not have been detected.

Our experiments performed using RR as a trigger for exocytosis instead of Ca^{2+} influx, however, demonstrate that the secretory process per se can be directly facilitated by forskolin (Figure 4). We have previously shown that this agent triggers quantal transmitter release in a way independent from Ca^{2+} influx or internal Ca^{2+} stores, by binding to external sites on terminals (Trudeau et al., 1996). The precise molecular mechanism underlying RR-induced exocytosis remains to be determined. However, our data are consistent with an action of RR at a late step in the vesicle cycle, downstream of vesicle docking and Ca^{2+} influx. RR may either bind to membrane phospholipids (Voelker and Smejtek, 1996) and reduce the energy barrier for exocytosis, or bind to proteins at the surface of the synaptic terminal and directly destabilize the vesicle docking complex. Whichever mechanism is operational, the observation that RR-evoked exocytosis is blocked by TeTx (Figure 3) demonstrates that its action depends on the integrity of synaptobrevin within the protein complex that is required for regulated secretion in neurons (Südhof, 1995). This agent can therefore be used as an assay of the state of the secretory machinery in synaptic terminals.

Our observation of a presynaptic enhancement of secretion by PKA could be mediated by any of three general classes of mechanisms. First, there could be an enhancement of the number of functional terminals, assuming some "silent" terminals existed before forskolin application. This hypothesis cannot be discounted, but is made unlikely by our observation that the average

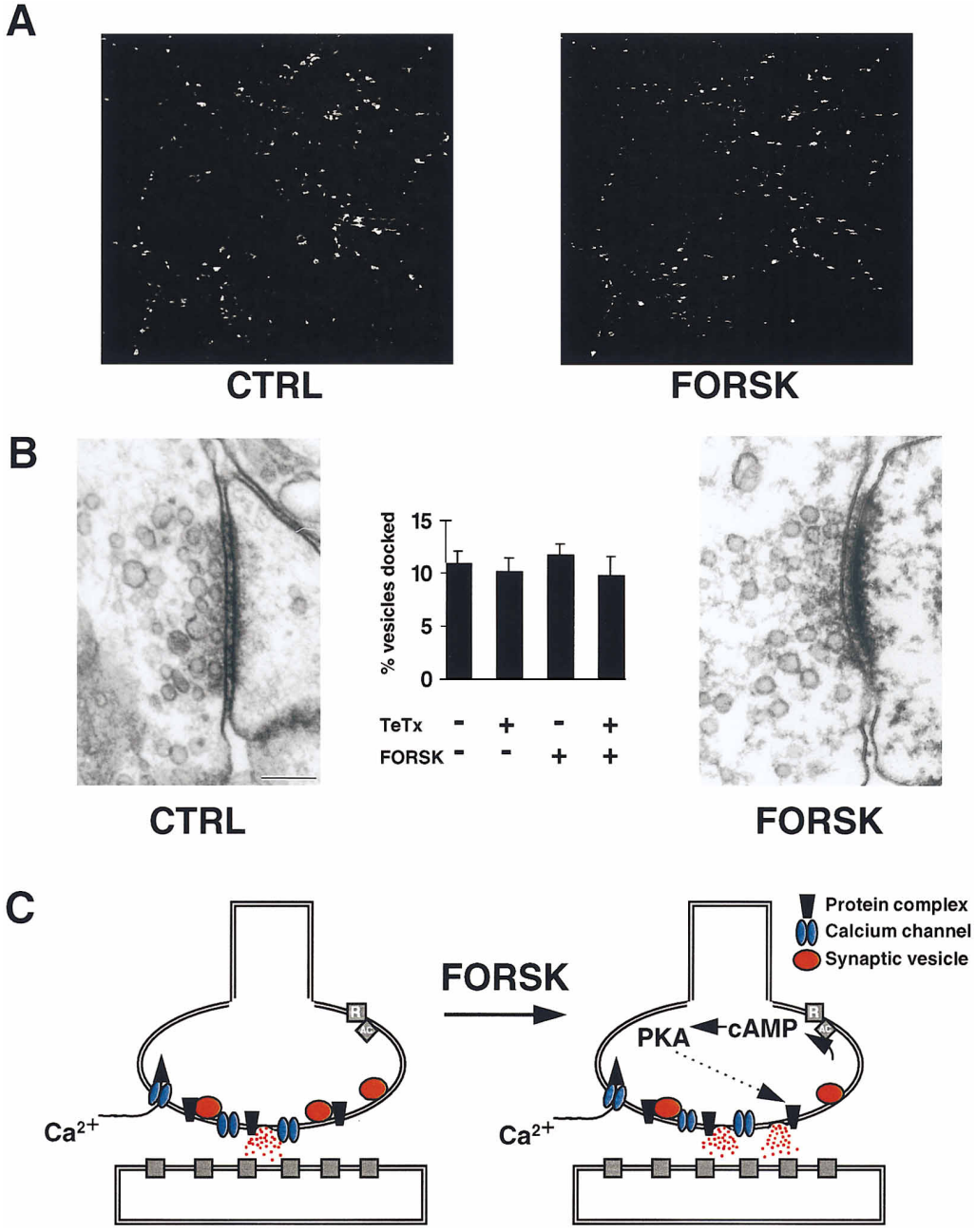

Figure 5. On the Mechanism of Facilitation of the Secretory Process by Forskolin

(A) Staining pattern of living synaptic terminals with the fluorescent indicator of synaptic vesicle recycling FM1-43. Active terminals were identified under control conditions and then again after 3 min in 20 μM forskolin (as described in Experimental Procedures). No significant difference in the number of stained terminals were detected. Calibration: 20 μm.

(B) Analysis by electron microscopy of synaptic terminals exposed to 20 μM forskolin. Sections from cultures treated with forskolin (FORSK), with or without pretreatment with TeTx to block regulated exocytosis, did not display an enhancement in the number or percentage of vesicles in contact with the presynaptic membrane (% vesicles docked). Thus, an increase in vesicle docking is unlikely to underly the synaptic facilitation produced by forskolin. Terminals treated with forskolin, however, displayed a small decrease in total number of vesicles per section (data not shown). Calibration: 130 nm.

(C) Model of the synaptic facilitation produced by forskolin. Following activation of adenylyl cyclase (AC) with forskolin, cAMP accumulation leads to activation of PKA in the presynaptic terminal. The kinase then phosphorylates one or more substrate proteins on synaptic vesicles or the presynaptic membrane. This reversible phosphorylation then directly facilitates the secretory process downstream from Ca^{2+} entry in the terminal, which is the usual trigger for exocytosis of neurotransmitter-filled vesicles.

number of functional terminals in any given field in our cultures, as determined by activity-dependent uptake of FM1-43 in recycling vesicles, is unchanged by forskolin (Figure 5A). A second class of mechanism could implicate a forskolin-induced enhancement of the number of morphologically "docked" vesicles in synaptic terminals. To explain our physiological observations, an approximate 2-fold enhancement would be expected. Our examination of synaptic terminals by electron microscopy does not support such a mechanism (Figure 5B), although it cannot be disputed that a small increase in vesicle docking could easily be undetectable under our conditions. The third class of mechanism could involve a forskolin-induced enhancement of the probability of exocytosis of previously docked vesicles (Figure 5C). This model requires one to make the assumption that vesicles observed to be "morphologically" docked are not necessarily "functionally" docked, as previously suggested (Schweizer et al., 1995) and confirmed by our ultrastructural observations of TeTx-treated synapses. This type of mechanism could explain the bulk of our observations. It would predict that a facilitation of action potential–evoked neurotransmitter release could occur even under conditions where Ca^{2+} influx is not enhanced (Figure 2C). It would also be compatible with our observation that the secretory response triggered by an agent that initiates exocytosis through an interaction with the extracellular presynaptic membrane (RR) could also be enhanced (Figure 4), under conditions where both vesicle docking and the number of active terminals is constant (Figures 5A and 5B). This model would also be compatible with the previous finding that the frequency of spontaneous mIPSCs in slice cultures of hippocampus is enhanced by forskolin (Capogna et al., 1995).

Our finding that the facilitation of action potential–evoked IPSCs was rapidly reversible upon washout of forskolin (Figure 1B) is intriguing in light of the previous observation that, at excitatory synapses formed onto CA3 pyramidal neurons of the hippocampus (Weisskopf et al., 1994) and at the crayfish neuromuscular junction (Dixon and Atwood, 1989), the facilitation of synaptic responses by this agent is relatively long lasting (>20 min). This is not likely to be due to washout of any soluble presynaptic factors within the presynaptic whole-cell pipette because we have observed the same rapid reversal in experiments where the presynaptic neuron was stimulated extracellularly (data not shown). One possibility is a differential basal or Ca^{2+}-induced phosphatase activity in GABAergic and glutamatergic nerve terminals, which would allow substrates phosphorylated by PKA to remain in that state for a longer period in the latter type of terminals. Alternatively, the facilitation produced by PKA in these different classes of terminals may involve the phosphorylation of different proteins.

The finding that the secretion of insulin from pancreatic β cells is similarly facilitated by PKA at a step apparently independent of Ca^{2+} elevation suggests that similar mechanisms may be involved in the control of regulated secretion in non-neuronal cells (Ämmälä et al., 1993). This possibility is also supported by the observation that in chromaffin cells and in PC12 cells, Ca^{2+}-dependent secretion can be "primed" by ATP (Bittner and Holz, 1992; Hay and Martin, 1992; Parsons et al., 1995). The mechanism has recently been shown to involve phosphorylation of phosphoinositides by a phosphatidylinositol-4-phosphate 5-kinase (Hay and Martin, 1993, 1995). Phosphorylation events dependent on protein kinase C (PKC) also appear to facilitate a late step in the secretory process in chromaffin cells (Gillis et al., 1996). Identifying the specific protein(s) involved in such PKA- and PKC-dependent events in neurons and other cell types could allow a better understanding of some of the limiting steps in the secretory process.

Previous reports have shown that neurotransmitter release by nerve terminals can be modulated by altering the influx of Ca^{2+} in presynaptic terminals (McGehee et al., 1995; Dittman and Regehr, 1996; Huang et al., 1996), presynaptic excitability (Byrne and Kandel, 1996), or postsynaptic responsiveness to neurotransmitters (Greengard et al., 1991; Rosenmund et al., 1994), illustrating the diversity of mechanisms available to finely regulate synaptic transmission. Previous reports showing that the frequency of spontaneous miniature synaptic currents can change in parallel to synaptic modulation have raised the possibility that the secretory machinery could also be directly regulated (Scholz and Miller, 1992; Scanziani et al., 1992; Malgaroli and Tsien, 1992). The direct modulation of the secretory process by PKA characterized here identifies an additional, potentially widespread mechanism involved in the modulation of neuronal communication.

Experimental Procedures

Neuronal Cultures and Electrophysiology

Postnatal mixed neuron/astrocyte hippocampal cultures were prepared as previously described (Basarsky et al., 1994). For dual whole-cell recordings, standard whole-cell patch-clamp recordings were obtained from the neurons using two Axopatch 1-D amplifiers (Axon Instruments, Foster City, CA). The normal internal solution was 140 mM K-gluconate, 10 mM EGTA, 4 mM Mg–ATP, 0.2 mM Tris–GTP, 10 mM HEPES (pH 7.35). The external saline contained higher concentrations of $MgCl_2$ to decrease spontaneous synaptic activity in the cultures. It was 135.5 mM NaCl, 7 mM $MgCl_2$, 3 mM $CaCl_2$, 5 mM KCl, 10 mM HEPES, 8 mM glucose (pH 7.35). The reversal potential of the IPSCs was found to stabilize around −75 mV within a few minutes after obtaining a whole-cell recording. It was monitored periodically during the course of the experiments. The data were analyzed using the pClamp software from Axon Instruments. For the recording of mIPSCs and mEPSCs (minis), the internal recording solution consisted of 117.5 mM Cs-gluconate, 10 mM NaCl, 4 mM $MgCl_2$, 5 mM EGTA, 2 mM Mg–ATP, 0.2 mM, Tris–GTP, 15 mM HEPES (pH 7.3). The reversal potential for the mIPSCs stabilized at about −37 mV within a few minutes after obtaining whole-cell recording and was checked periodically within the course of the experiments. To establish the cumulative probability plots, the minis (recorded in 15 s episodes) were normalized to the median mini amplitude of the control episode of each experiment. The normalized mini amplitudes were then ranked and divided in 0.1 unit bins for the subsequent establishment of a cumulative probability plot. The probability determined for each bin was averaged across six experiments. The points represent the mean ± SEM for each bin. Miniature synaptic currents were analyzed with software provided by Dr. P. Vincent (University of California, San Diego). Experiments investigating the effect of forskolin on action potential–evoked release and RR-evoked release were performed on different preparations because of the requirement to block action potentials with TTX to isolate miniature synaptic events, and also because RR blocks Ca^{2+} channels in nerve terminals (Trudeau et al., 1996).

Ca^{2+} and FM1-43 Imaging

$[Ca^{2+}]_i$ levels were determined through standard techniques by loading the cells with fura-2–AM. Images were acquired as previously

described (Basarsky et al., 1994) using a CH-250 cooled CCD camera (Photometrics, Tucson, AZ) and Metafluor software (Universal Imaging, West Chester, PA). For FM1-43 staining experiments, the images were collected on an Odyssey scanning laser confocal microscope (Noran Instruments, Middleton, WI). The FM1-43 was excited using the 488 nm line of the laser. The emission filter had a cutoff of 515 nm. Images were analyzed using Image 1/AT software (Universal Imaging). Coverslips were mounted in a laminar flow chamber and imaged through a 60× plan apo oil immersion objective (1.40 NA; Nikon, New York, NY). The neurons were depolarized with saline containing 40 mM K^+ and 10 μM FM1-43 for 60 s. A second depolarization permitted release of the sequestered indicator. The uptake and release of FM1-43 was found to be dependent on depolarization and Ca^{2+} influx as previously shown for hippocampal neurons (Ryan et al., 1993; Reuter, 1995). Digital subtraction of the signal detected after loading from that obtained after release of the dye with the second depolarization allowed the detection of functional terminals. This protocol was performed twice for each preparation: once under control conditions and then again after 3 min in 20 μM forskolin.

Electron Microscopy

After 90 min fixation (1% paraformaldehyde, 2% glutaraldehyde, and 0.25% tannic acid in 0.1 M cacodylate buffer), cultures were postfixed for 15 min in 0.5% osmium tetroxide plus 0.8% potassium ferricyanide in cacodylate buffer. After en-block staining with Mg–uranyl acetate (1%, aqueous, 2 hr), cultures were embedded in Epon resin. Thin sections were stained with Reynolds' lead citrate before viewing. Only sections that displayed a clear synaptic specialization were selected. The data was analyzed using a blind procedure.

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