Regulation of the Readily Releasable Vesicle Pool by Protein Kinase C

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Summary

Modulation of the size of the readily releasable vesicle pool has recently come under scrutiny as a candidate for the regulation of synaptic strength. Using electrophysiological and optical measurement techniques, we show that phorbol esters increase the size of the readily releasable pool at glutamatergic hippocampal synapses in culture through a protein kinase C (PKC)–dependent mechanism. Phorbol ester activation of PKC also increases the rate at which the pool refills. These results identify two powerful ways that activation of the PKC pathway may regulate synaptic strength by modulating the readily releasable pool of vesicles.

Introduction

In synapses, the readily releasable pool is the population of neurotransmitter-filled vesicles that are immediately available for release. Recent experiments have determined some of the properties of the readily releasable pool of synaptic vesicles (Borges et al., 1995; Ryan and Smith, 1995; Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996; Dobrunz and Stevens, 1997; Murthy et al., 1997; von Gersdorff and Matthews, 1997; see also Wang and Zucker, 1998). In practice, this pool is defined as the maximum number of vesicles that can be released in 2 or 3 s and is thought to coincide with those vesicles that are docked to the active zone and primed for release. The size of the readily releasable pool is likely to be an important factor contributing to the probability of release at a synapse. Our goal here is to identify a factor that regulates the size of the readily releasable pool. To place this goal in context, we will briefly review properties of this pool.

The average pool size for hippocampal CA1 synapses is about 5–10 vesicles (Harris and Sultan, 1995; Stevens and Tsujimoto, 1995; Dobrunz and Stevens, 1997; Schikorski and Stevens, 1997; Murthy and Stevens, 1998). The resting probability of release at a synapse is approximately linearly related to the pool size (Dobrunz and Stevens, 1997; Murthy et al., 1997). As the vesicle pool is depleted—for example, during high-frequency stimulation—release probability declines correspondingly (Dobrunz and Stevens, 1997). Every time a vesicle is released, a docking site is left unavailable for immediate use; we term such sites "empty" and refer to their return to a release-ready state as "refilling," although the actual

mechanism may be more complicated. Empty sites are refilled with a time constant of about 5–10 s (Stevens and Tsujimoto, 1995; Dobrunz and Stevens, 1997; von Gersdorff and Matthews, 1997), although the exact refilling rate can vary severalfold from one cell to another and from one time to the next in the same cell, depending on the history of synaptic use (von Gersdorff and Matthews, 1997; Stevens and Wesseling, 1998; Wang and Kaczmarek, 1998; see also Brodin et al., 1997). Although the size of the readily releasable pool at rest is unaffected by a range of manipulations that alter release probability, it is decreased by long-term depression in culture (Goda and Stevens, 1998).

We sought a manipulation that could increase the size of the readily releasable pool. A candidate manipulation is suggested by the observation of Neher and colleagues (Gillis et al., 1996) that the readily releasable pool of secretory granules in chromaffin cells is increased by activation of protein kinase C (PKC). Although the relationship between granule pools in chromaffin cells and vesicle pools at synapses is unclear, PKC activation seemed to be a good candidate for pool regulation at mammalian synapses. Phorbol esters, which directly activate PKC by binding to this kinase's regulatory "C1" domain (Castagna et al., 1982; Newton, 1997), have been shown to increase release probability (Malenka et al., 1986; Shapira et al., 1987; Segal, 1989), and several synaptic proteins have PKC phosphorylation sites.

We find that phorbol esters do increase the size of the readily releasable pool at glutamatergic hippocampal CA1 and CA3 synapses in culture through a PKC-dependent mechanism. This increase is likely to contribute to the potentiation of synaptic responses observed following phorbol application. Phorbol ester treatment also increases the rate at which empty sites in the pool are refilled. Recent results show that calcium influx associated with electrical activity also accelerates the refilling of empty sites in the readily releasable pool (Stevens and Wesseling, 1998; Wang and Kaczmarek, 1998), but the PKC-dependent mechanism described here, while sharing a common final pathway, is distinct from this process.

Results

Activation of PKC Increases the Size of the Readily Releasable Vesicle Pool
Electrophysiological Measurements
To test the hypothesis that PKC activation increases the size of the readily releasable pool, we measured the size of this pool in individual cultured hippocampal neurons before and after application of phorbol esters. We exclusively studied excitatory (glutamatergic) autaptic neurons. Using whole-cell recording, we estimated the readily releasable pool size by applying hypertonic solution to these neurons and measuring the total charge transferred by synaptic currents (see Experimental Procedures). Application of hypertonic solution empties the readily releasable pool through a Ca^{2+}-independent release mechanism (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996), so the size of the synaptic

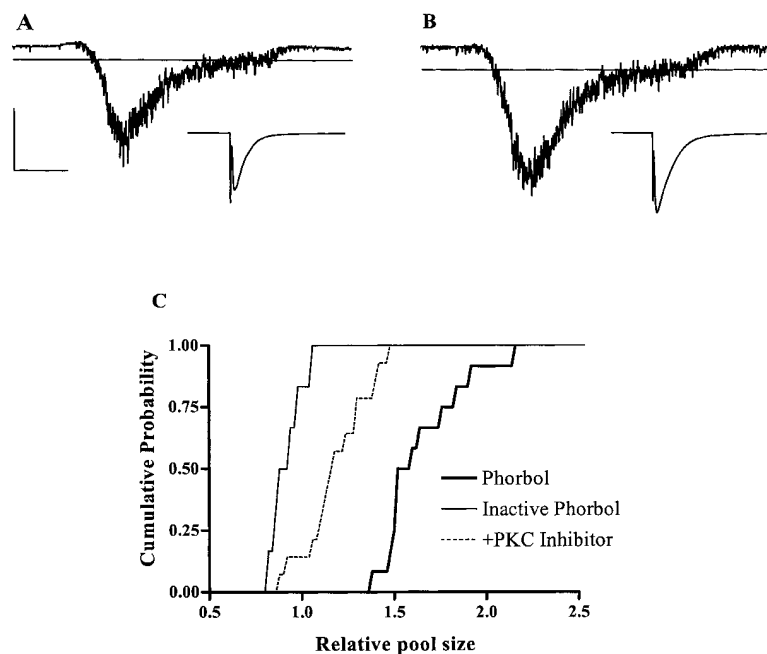‡To whom correspondence should be addressed (e-mail: janes@axp2.salk.edu).

Figure 1. Activation of PKC Increases the Size of the Readily Releasable Vesicle Pool Measured Electrophysiologically by Application of Hypertonic Solution

(A) Whole-cell response from an autaptic cultured rat hippocampal neuron evoked by a 4 s application of hypertonic solution. Following an initial peak in release, the response declines to a low steady-state level, marked by the horizontal line. This steady-state response is believed to reflect release of vesicles that are being drawn from the reserve pool of vesicles several microns away from the active zone, and it was subtracted from the current integral calculations used to estimate the size of the readily releasable pool. The inset shows the autaptic response—a fast sodium spike (action potential) followed by an EPSC—evoked in this neuron by a brief depolarizing pulse from −60 mV to −20 mV. Scale bars, 500 pA/6000 pA and 1 s/50 ms for responses to hypertonic solution application/EPSCs.

(B) Response from the same neuron during bath application of the phorbol ester PMA (0.1 μM). There was a 1.51-fold increase in the size of the readily releasable pool and an approximate doubling of the size of the steady-state response. The inset shows a 1.67-fold increase in the size of the EPSC.

(C) Cumulative probability distribution of the change in pool size in each neuron following application of phorbol ester PMA, PDA, or PDBu (thick solid line), phorbol in the presence of PKC inhibitor staurosporine, bisindolymaleimide I, or H-7 (dashed line), or inactive phorbol 4α-PMA (thin line), expressed as value normalized to prephorbol control. Mean increases (± SEM) were 1.65 ± 0.07 times control for the phorbol group (n = 12); 1.19 ± 0.05 (n = 14) for the PKC inhibitor group; and 0.92 ± 0.04 (n = 6) for the inactive phorbol control group. See Experimental Procedures for methodological details.

response to application of hypertonic solution reflects the number of vesicles in the pool. Provided there is no change in the postsynaptic sensitivity to glutamate, a change in the charge transferred by the synaptic response to hypertonic application indicates a change in the size of the readily releasable vesicle pool.

Phorbol application increased the size of excitatory postsynaptic currents (EPSCs) recorded in cultured hippocampal neurons (1.96 ± 0.17 times the control value, range 1.39–3.45; n = 12; Figure 1A and 1B, insets), as described previously (Segal, 1989; Goda et al., 1996). In these same cells, the size of the response to application of hypertonic solution (Figures 1A and 1B) was increased by 1.65 ± 0.07 times the control (range 1.37–2.15; Figure 1C). Phorbol application had no effect, however, on the amplitude of spontaneous miniature EPSCs (mEPSCs; 1.06 ± 0.06 times the control; n = 7), measured in the presence of tetrodotoxin (TTX; 2 nM) to inhibit spontaneous action potential firing. Phorbols did increase the frequency of mEPSCs by 6.06 ± 1.57 fold. The average frequency of mEPSCs prior to phorbol application was 2.7 ± 1.7 per second and increased to 9.2 ± 3.5 after phorbol application. The effects we observed on mEPSC amplitude and frequency are comparable to those described previously for hippocampal neurons in slices (Malenka et al., 1986; Shapira et al., 1987; Parfitt and Madison, 1993) and in culture (Finch and Jackson, 1990; Goda et al., 1996). Because spontaneous mEPSCs are believed to be the postsynaptic response to a single spontaneously released synaptic vesicle, the lack of effect of PKC on mEPSC amplitude indicates that there

was no PKC-mediated change in the sensitivity of postsynaptic receptors to glutamate. We conclude that phorbol application increased the size of the readily releasable vesicle pool.

To determine whether the effects of phorbol esters on pool size were due to the activation of the PKC signaling pathway, we applied phorbols after incubation with inhibitors of PKC. PKC inhibitors act by binding to the catalytic, ATP-binding domain of the kinase (Toullec et al., 1991), a site distinct from the regulatory "C1" domain to which phorbol esters bind. Application of phorbol had a much smaller effect on the synaptic function of cells that had been incubated in extracellular solution containing PKC inhibitors. Cells (n = 14) that were incubated in the presence of PKC inhibitors staurosporine (1 μM), bisindolymaleimide I (1 μM), or H-7 (100–300 μM) for at least 30 min showed only a 1.19 ± 0.06 fold change in EPSC size (range 0.84–1.56) and a 1.19 ± 0.05 fold change in pool size (range 0.88–1.48; Figure 1C). Thus, the increase in pool size with phorbol ester application appears to require the function of PKC.

To control for nonspecific, PKC-independent actions of phorbols (and the vehicle DMSO), we tested the effects of 4α-PMA, a phorbol that does not activate PKC. Application of 4α-PMA in DMSO (n = 6) had no effect on the size of the EPSC (0.91 ± 0.08 times control; range 0.53–1.06) or the readily releasable pool (0.92 ± 0.04 times control; range 0.82–1.05; Figure 1C), although all of these cells showed an increase in the size of the EPSC (1.92 ± 0.27 fold change) and the readily releasable pool (1.48 ± 0.10 fold change) in response to subsequent

application of active phorbol. In these cells, the small decrease in the sizes of the EPSC and the readily releasable pool following application of 4α-PMA reflects the "run down" that normally occurs in patch clamped cells with time (Goda et al., 1996; Goda and Stevens, 1998; Stevens and Wesseling, 1998). These data together indicate that activation of PKC increases neurotransmitter release—as evidenced by an increase in EPSC size without a concomitant change in mEPSC amplitude—and that this increase is due, at least in part, to an increase in the size of the pool of readily releasable vesicles.

Optical Measurements

To examine how PKC activation affects the readily releasable pool at individual synapses in cells that had not been subjected to patch clamping, and also to confirm the results presented above with a completely different method, we measured the effect of phorbol esters on pool size optically, using the fluorescent membrane dye FM1-43 (Betz and Bewick, 1992; Ryan and Smith, 1995; Murthy and Stevens, 1998). For these experiments, performed on cultured hippocampal neurons, the readily releasable pool was fully emptied with 20–40 action potentials (Rosenmund and Stevens, 1996; Dobrunz and Stevens, 1997; Murthy and Stevens, 1998) delivered in the presence of FM1-43. Our experience is that estimates of the pool size are almost independent (within 10%–20%) of the number of stimuli between 20 and 80 action potentials (V. Murthy and C. F. S., unpublished observations). As the released vesicles were retrieved (endocytosed), fluorescent dye was taken up at each release site. After subtracting whatever fluorescence remained after destaining with 1000 nerve impulses, the fluorescent intensity at each site was thus proportional to the size of the readily releasable pool at that synapse. Fluorescent intensity was then divided by the value for single vesicle fluorescence to yield an estimate of the number of vesicles in the readily releasable pool (Murthy and Stevens, 1998).

Because any spontaneous action potentials during the 50 s dye-loading phase will increase the apparent size of the readily releasable pool, phorbol-mediated changes in excitability (Baraban et al., 1985; Hoffman and Johnston, 1998) could conceivably affect the size of the pool during optical measurement. To ensure that changes in excitability were not responsible for any measured changes in pool size, in one experiment we added 1 μM TTX to the FM1-43 containing solution immediately after delivering the train of action potentials that were used to unload and stain the readily releasable pool. Data from this experiment were similar to those performed in the absence of TTX, and all data were pooled.

What effect might the large increase in spontaneous mEPSC frequency have on our FM1-43 results? The increase in spontaneous vesicle release during the dye-loading phase could cause an overestimate of pool size, but it will also increase the release of dye-loaded vesicles during the 5 min wash phase prior to imaging (see Experimental Procedures), leading to an underestimate of pool size. To ensure that phorbol effects on spontaneous release were not contributing significantly to any optically measured changes in pool size, we stained synapses in the presence of 1 μM TTX without applying the train of action potentials normally used to unload and stain the readily releasable pool, and then we destained with 1000 action potentials. We repeated this procedure before and after application of phorbol. At 41 synapses that were stained by spontaneous loading before application of phorbol (two separate experiments), the average number of vesicles was 2.0 ± 0.2; at 49 synapses that spontaneously loaded after phorbol the average number of vesicles was 2.2 ± 0.2. These numbers indicate that while spontaneous loading accounts for some portion of the optically measured readily releasable pool, there is little difference in this contribution before and after phorbol application. In addition, it should be kept in mind that spontaneously stained synapses represent only about 20%–25% of the total stainable synapses in any field (V. Murthy, personal communication). Therefore, the number of spontaneously loaded vesicles contributing to our optically measured pool at each synapse is only about 0.5 vesicles per synapse on average.

Fluorescent intensity was measured at a total of 44 synapses that stained and destained before and after phorbol application in three separate experiments (Figures 2A and 2B). The number of vesicles in the readily releasable pool at these synapses was 5.2 ± 0.3 before phorbol and increased to 8.5 ± 0.7 after phorbol application (Figure 2C), reflecting a 1.63-fold increase in pool size. Application of 4α-PMA (1 μM) in DMSO had no effect on the size of the readily releasable pool measured in 48 synapses: 7.5 ± 0.5 before 4α-PMA and 6.8 ± 0.5 after 4α-PMA (0.91 times control). Optical measurements at individual synapses thus confirm our electrophysiological findings that active phorbol application increases the size of the readily releasable vesicle pool by about 1.6-fold.

Activation of PKC Speeds up the Rate at which the Pool Is Refilled

In addition to increasing the size of the vesicle pool, activation of PKC also increased the rate at which the pool refilled following depletion. The refilling rate was measured by emptying the pool twice in succession using hypertonic solution (see Figures 4A and 4B). The ratio of the size of the response to the second application relative to that of the first gave a measure of how much the pool refilled during the time between the first and second applications (Stevens and Tsujimoto, 1995; Stevens and Wesseling, 1998). The time course of refilling was obtained by systematically varying the time between hypertonic challenges. By repeating this protocol before and after phorbol application, we determined the effect of PKC activation on the rate at which the pool refilled after being emptied.

We found an approximate doubling of the refilling rate after phorbol application. For simplicity, we fit the time course of refilling with a single, rather than double, exponential time constant. This time constant was 9.1 s before phorbol application and sped up to 4.8 s after phorbol application (Figure 3). The phorbol-mediated increase in the rate of refilling, measured at 2.5 s, was quite effectively blocked by PKC inhibitor bisindolymaleimide I (1 μM; Figure 3), suggesting that the increased rate was mediated by PKC. PKC inhibition has recently been reported to speed up the rate at which vesicles are

A

B

1μm

C

Cumulative Probability

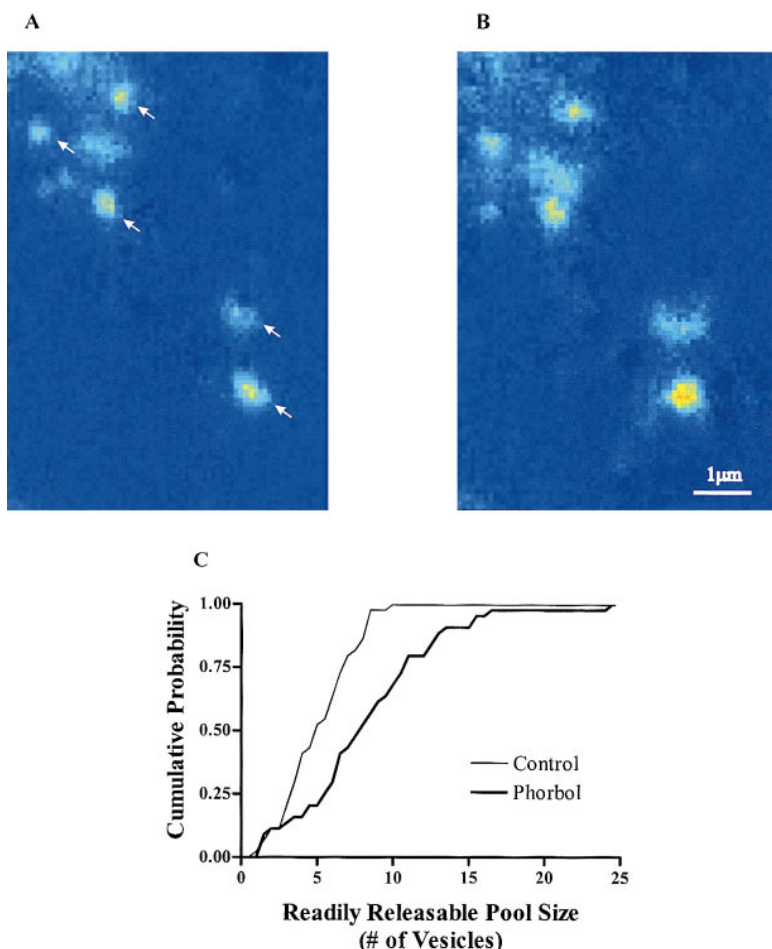Readily Releasable Pool Size
(# of Vesicles)

— Control
— Phorbol

Figure 2. Activation of PKC Increases the Size of the Readily Releasable Vesicle Pool Measured Optically by FM1-43 Labeling

(A) Fluorescence image of a field of neuronal processes after loading with FM1-43.

(B) Fluorescence image of the same field loaded with FM1-43 after bath application of 1 μM PMA for 2 min. In this field, five synapses (marked by arrows in [A]) stained and destained before and after phorbol application. The mean pool size at these synapses was 5.9 ± 0.9 vesicles before phorbol and 9.1 ± 1.3 vesicles after phorbol treatment, reflecting a 1.54-fold increase in the readily releasable pool size.

(C) Cumulative probability distribution of the readily releasable pool size at each synapse before phorbol (1 μM PMA; thin line) and after application of active phorbol (thick solid line). Mean pool size was 5.2 ± 0.3 vesicles before phorbol (n = 44) and 8.5 ± 0.7 after treatment with active phorbol. See Experimental Procedures for methodological details.

endocytosed (Klingauf et al., 1998). This finding may seem inconsistent with the results presented here, but it is important to keep in mind that endocytosis and refilling are separate processes: refilling most likely reflects a relatively rapid replenishment of vesicles from a reserve pool of vesicles, while recently endocytosed vesicles require approximately 40 s to become available for release (Ryan et al., 1993). Although an increase in the rate of refilling will not contribute to the amount of neurotransmitter released by an isolated single action potential, it does have implications for the amount of transmitter that can be released during a high-frequency train of action potentials (see Discussion).

The Mechanism Underlying the Increase in Refilling Rate following PKC Activation Is Not the Same as that following Tetanic Stimulation—but They Share a Common Final Pathway

The rate at which the readily releasable vesicle pool refills also increases after a high-frequency train of action potentials (Stevens and Wesseling, 1998; Wang and Kaczmarek, 1998). This acceleration in refilling depends on the accumulation of intracellular Ca^{2+} during electrical stimulation. To determine whether this Ca^{2+}-dependent increase in the refilling rate shares a common mechanism with the PKC-dependent increase, we measured the effect of electrical stimulation on the rate of

refilling before and after phorbol application. For these whole-cell-recording experiments, a pair of hypertonic challenges was delivered 2.5 s apart. Trials in which there was no electrical stimulation were interleaved with trials in which a train of 14 action potentials was elicited at 9 Hz during the final 1.5 s of the first hypertonic challenge (Figure 4A). Perforated patch recordings were used to minimize interference with endogenous calcium buffering at the terminal.

The rate of refilling was 1.65 ± 0.14 times faster (n = 6) for trials in which action potentials were elicited at the end of the first hypertonic solution application (Figures 4A and 4C) than for those with no electrical stimulation, as described by Stevens and Wesseling (1998). After phorbol application, the rate of refilling was only 1.12 ± 0.30 times faster for those trials in which action potentials were elicited (Figures 4B and 4C). Thus, phorbol application and nerve impulse activity both speed the refilling, and the increased refilling rate produced by phorbol application occludes that resulting from action potentials. We interpret this occlusion as indicating a shared pathway between the two mechanisms.

One might suppose from the above results that the effects of electrical stimulation are due to PKC activation by calcium entering and accumulating in the nerve terminal. However, application of the PKC inhibitors bisindolymaleimide I (1 μM) or staurosporine (1 μM) failed to
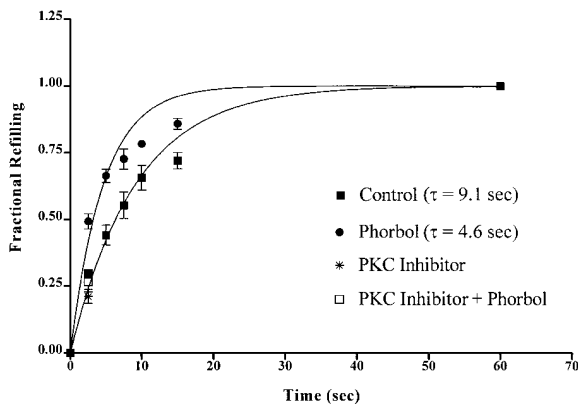
Figure 3. Activation of PKC Speeds up the Rate at which the Readily Releasable Vesicle Pool Refills after Depletion

The time course of recovery of the readily releasable pool was measured after emptying the pool with an application of hypertonic solution. A second application was begun 2.5–15 s after the first ended (see Figures 4A and 4B). The fractional refilling was calculated as the size of the response to the second hypertonic solution application (corrected for steady-state exocytosis) relative to the corrected response to the first application. Each point (mean ± SEM) represents data from 6–16 neurons; not all neurons were used for measurements at every time point, nor were all neurons used both before and after phorbol application. The refilling was fit by a single exponential with a time constant of about 9 s for cells before phorbol application (squares), and about 4.5 s after phorbol application (circles). Also included are data points showing the amount of refilling measured at 2.5 s in the presence of the PKC inhibitor bisindolymaleimide I ($1 \mu M$) before and after application of phorbol (PMA, $1 \mu M$; n = 8).
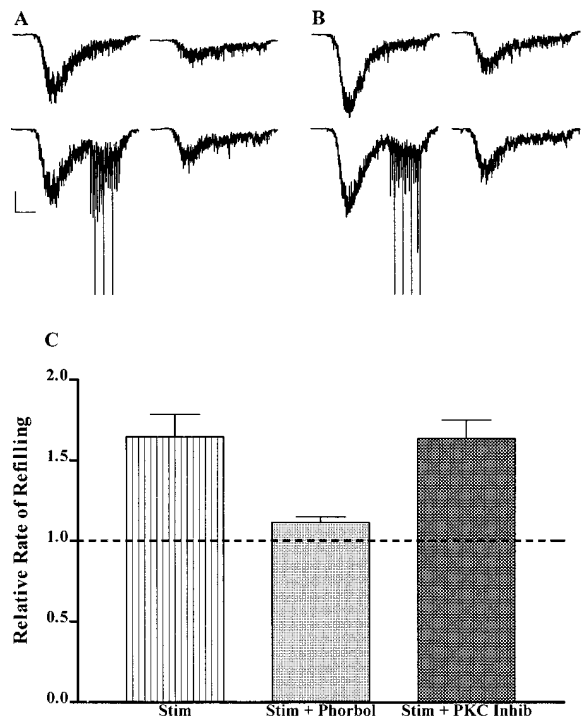

Figure 4. PKC Activation and Tetanic Stimulation Both Speed up the Refilling Rate of the Readily Releasable Vesicle Pool by Acting through a Common Final Pathway, but They Do Not Share the Same Mechanism of Action

(A) Whole-cell responses from a cultured rat hippocampal neuron evoked by a pair of 5 s long hypertonic challenges applied 2.5 s apart. Upper traces, no stimulation; lower traces, with a train of 14 sodium spikes (action potentials) elicited during the last 1.5 s of the first hypertonic challenge (of each pair). Because of the slower sampling rate (200 Hz) used to acquire data during hypertonic challenge, many of the fast sodium spikes are not seen in their entirety; larger spikes were truncated for clarity. The rate of refilling of the pool was increased 1.5-fold by stimulation. Scale bars, 250 pA and 1 s.

(B) After phorbol application, the pool size increased, and the amount of refilling during the 2.5 s between applications in the absence of stimulation was doubled. However, the addition of electrical stimulation now increased this rate by only 1.1-fold.

(C) Average increases (mean ± SEM) in the rate of refilling due to electrical stimulation before and after application of $1 \mu M$ PMA (1.65 ± 0.14 and 1.12 ± 0.30, respectively; n = 6). Although these data suggest that PKC activation and electrical stimulation act through a common final pathway, electrical stimulation still sped up the rate of refilling in the presence of PKC inhibitor (1.64 ± 0.11; n = 6). This result indicates that electrical stimulation does not affect the rate of refilling by activating PKC.

block the electrically induced increase in refilling rate (1.64 ± 0.11 times faster than control; n = 4; Figure 4C). Subsequent application of phorbol had no effect on the size of the EPSCs in these cells, confirming that PKC was indeed inhibited in these cells. We conclude that, while sharing a common final pathway, these two mechanisms are at least partially distinct and that the increased refilling rate seen with a burst of action potentials is not mainly due to PKC activation.

Discussion

We have found that phorbol application increases the size of the readily releasable vesicle pool in cultured hippocampal neurons. Electrophysiological measurements during hypertonic solution application showed a 1.65-fold increase in the size of this pool, while optical measurements using FM1-43 showed a 1.63-fold increase. This phorbol-mediated increase in pool size was much reduced in the presence of PKC inhibitors, and 4α-PMA, a phorbol that does not activate PKC, had no effect on pool size. We interpret these observations to mean that activation of PKC increases the size of the readily releasable vesicle pool by about 1.6-fold.

What mechanisms underlie this change in the size of the readily releasable pool? Activation of PKC by phorbol esters has previously been shown to cause disassembly of a subplasmalemmal network of actin filaments (Vitale et al., 1992, 1995) and to increase the number of

morphologically docked secretory granules in chromaffin cells (Vitale et al., 1995). A similar mechanism could produce the PKC-mediated increase in the readily releasable pool in cultured hippocampal neurons. PKC activation could also alter the phosphorylation state and, consequently, the function of proteins involved in the docking and release of synaptic vesicles. For example, SNAP-25 and munc18/nSec1 are two synaptic proteins phosphorylated by PKC (Fujita et al., 1996; Shimazaki et al., 1996), making them attractive candidate agents of the PKC-mediated increase in pool size. One means by which these proteins could act is suggested

by the observation that only about half of the potential docking sites at the active zone are usually occupied (Schikorski and Stevens, 1997); that is, the active zone area would accommodate about twice as many vesicles if every docked site were filled. This hypothesis implies that a synapse could increase its pool size without major changes in synaptic structure by regulating the availability of empty docking sites. One could thus imagine that "blocked" or silent sites are made available to vesicles by phosphorylation of a protein such as munc18/nSec1.

The increase in charge transfer that we observe following phorbol application is not the result of a change in the postsynaptic responsiveness to glutamate. PKC activation did not change the amplitude of mEPSCs, in agreement with a host of previous studies (Malenka et al., 1986; Shapira et al., 1987; Finch and Jackson, 1990; Parfitt and Madison, 1993; Goda et al., 1996). Altogether, these findings strongly discount the idea that the observed increase in EPSC amplitude and synaptic charge transfer produced by hypertonic challenge could reflect a change in glutamate sensitivity resulting from PKC phosphorylation of postsynaptic glutamate receptors.

Why did PKC inhibitors fail to completely suppress the phorbol-mediated increases in EPSC and pool size? One simple and likely possibility is that PKC inhibitors do not completely inhibit PKC in these cells. A more interesting alternative is that some of the inhibitor-resistant effects of phorbol esters we observed were due to the direct action of phorbols on proteins other than PKC. Phorbols activate PKC by binding to its diacylglycerol binding site, the so-called "C1" domain (Castagna et al., 1982; Newton, 1997). However, phorbols could also act by binding to other proteins that contain this "C1" domain, such as the presynaptic protein munc13-1 (Brose et al., 1995; Betz et al., 1997), which enhances release of neurotransmitter upon binding phorbol ester (Betz et al., 1998).

The fullness of the readily releasable pool is directly related to the probability that neurotransmitter will be released (Rosenmund and Stevens, 1996; Dobrunz and Stevens, 1997; Murthy et al., 1997; see also Wang and Zucker, 1998). Therefore, the increase in the size of this pool following phorbol application should cause an increase in EPSC size, as is observed. However, the percent increase in the readily releasable pool was consistently smaller than the increase in EPSC size of the same cell. This result suggests that PKC activation has additional effects that contributed to the observed increase in synaptic strength. What might these additional effects be?

Phorbol esters have been reported to inhibit potassium currents in hippocampal neurons (Doerner et al., 1988; Hoffman and Johnston, 1998). Inhibition of potassium currents likely contributes to the enhancement of excitability observed in these cells after phorbol application (Baraban et al., 1985). Phorbol-mediated inhibition of potassium currents also broadens action potentials (Storm, 1987), which would be expected to increase calcium influx and, consequently, release probability and EPSC size. Such changes in excitability and/or action potential duration would not be expected to affect the size of the readily releasable pool when measured using

hypertonic solution application, because action potential–dependent release does not occur during these measurements.

What about the possibility that phorbol-mediated changes in potassium currents alter the electrotonic properties of hippocampal neurons and consequently produce changes in somatically recorded currents that do not reflect changes in synaptic responses? A phorbol-mediated reduction in sustained potassium currents of acutely isolated guinea pig hippocampal neurons has been reported (Doerner et al., 1988); such changes could alter the membrane time constant and lead to an increase in somatic current measurements that does not reflect a true increase in neurotransmitter release. However, in preliminary studies, we found no effect of phorbol esters on sustained potassium currents in our cultured hippocampal neurons (data not shown). The absence of a change in sustained potassium currents, as well as the lack of change in mEPSC amplitude, argues against the possibility that phorbol-mediated changes in membrane properties could account for our electrophysiologically measured increase in pool size. More recently, Hoffman and Johnston (1998) found that only the transient A-type potassium current was inhibited by PKC activation in rat hippocampal neurons. PKC-mediated inhibition of this rapidly ($<$100 ms) inactivating A current is probably not altering the cable properties of dendrites sufficiently over the several-second-long application of hypertonic solution to contribute significantly to our electrophysiologically measured increase in pool size. PKC-mediated changes in the A current may, however, lead to changes in excitability and spike broadening.

What effect might changes in excitability have on our optical measurements of pool size? The contribution of such changes was assessed by including TTX in the FM1-43 containing solution immediately after delivery of the train of action potentials used to unload and stain the pool. There was little difference in the increase in pool size measured in the presence and absence of TTX, indicating that changes in spontaneous firing are not likely to be responsible for the optically measured increase in pool size. What about the effects of spike broadening on our optical measurements? Because the 20–40 action potential train in our protocol is expected to cumulatively release all the vesicles in the readily releasable pool, no more vesicles could be released using this protocol, even with additional calcium influx during each action potential.

In summary, phorbol-mediated changes in potassium currents are not likely to contribute significantly to the increase in pool size measured either electrophysiologically or optically. Such changes could contribute to an increase in the amount of neurotransmitter released by a single action potential by broadening the action potential and increasing the amount of calcium entering the terminal. These changes may explain the discrepancy between the increase in the EPSC and the increase in the size of the readily releasable vesicle pool.

In addition to inhibiting potassium currents, phorbol esters enhance N- and L-type Ca^{2+} currents in rat hippocampal neurons (O'Dell and Alger, 1991; Parfitt and Madison, 1993; Swartz et al., 1993; but cf. Doerner et al., 1988). This enhancement would likely increase the

amount of calcium entering the presynaptic terminal upon invasion of an action potential and thus contribute to an increase of action potential–evoked neurotransmitter release. Because hypertonic solution empties the readily releasable pool by a Ca^{2+}-independent mechanism (Rosenmund and Stevens, 1996), and because pool size is unaffected by changes in intracellular calcium concentration (Rosenmund and Stevens, 1996; Stevens and Wesseling, 1998), phorbol effects on calcium influx should not influence pool size measured by hypertonic challenge. In FM1-43 studies, on the other hand, enhanced calcium influx could increase the amount of release evoked by each action potential in the train used to empty the readily releasable pool. However, because this train is expected to cumulatively release all the vesicles in the readily releasable pool, no more vesicles could be released, even with additional calcium influx. A phorbol-mediated enhancement of calcium currents is therefore not expected to affect either the electrophysiologically or optically measured pool size and could contribute to the discrepancy between the increase in the EPSC and the increase in the size of the readily releasable vesicle pool.

Phorbol-mediated changes in potassium and calcium currents may adequately explain the discrepancy between the measured changes in the size of the EPSC and the readily releasable vesicle pool, but they do not explain the discrepancy between the 6-fold increase in mEPSC frequency and the 1.6-fold increase in pool size. While an increase in pool size should increase mEPSC frequency, the magnitude of the effect ought to be comparable for both. Likewise, while the phorbol-mediated increase in calcium influx through L-type Ca^{2+} channels has been shown to contribute to the phorbol-mediated acceleration of the spontaneous mEPSC rate, it does not fully account for the magnitude of the change (Parfitt and Madison, 1993). These gaps suggest that phorbols have an additional effect on synaptic proteins involved in vesicular release (such as SNAP-25, munc18/nSec1, and/or munc13-1; see above) that leads to a lowering of the energy barrier for release of individual vesicles.

Finally, we have shown that activation of PKC by phorbol esters also increases the rate at which empty sites in the readily releasable vesicle pool are refilled, an effect recently reported for the secretory granule pool in chromaffin cells (Smith et al., 1998). What effect will this rate change have on synaptic transmission? The increase in the rate of refilling should not affect the amount of neurotransmitter released in response to a single action potential occurring after an extended period of quiescence (i.e., enough time that even the slowest filling pools would be completely refilled), but it should increase the amount of transmitter released during a long high-frequency train of action potentials. During the initial 1–3 s of high-frequency stimulation, the readily releasable vesicle pool is depleted (Rosenmund and Stevens, 1996; Dobrunz and Stevens, 1997). After depletion, the amount of neurotransmitter that can be released is limited by the rate at which vesicles are brought from the reserve pool to the readily releasable pool and should thus become insensitive to the rate of action potential firing. Indeed, Abbott et al. (1997) find that the steady-state synaptic response to a long, high-frequency train of action potentials is inversely proportional to the rate of stimulation. That is, once the steady state is achieved during high-frequency stimulation, the average amount of transmitter released per unit time is independent of the rate of stimulation and is instead predominantly a function of the rate at which empty sites in the pool can be refilled. A doubling of this rate would therefore double the amount of transmitter that can be released during a long high-frequency train.

We have identified activation of the PKC signaling pathway as the first known mechanism to increase the size of the readily releasable vesicle pool in neurons. We have also identified activation of the PKC pathway as the second known mechanism to increase the rate at which the pool refills following depletion. Both of these mechanisms provide a powerful means to modulate synaptic strength. Additional regulatory mechanisms must be identified and studied before we fully understand how the size and fullness of the readily releasable pool regulates the efficacy of excitatory synaptic transmission in the central nervous system.

Experimental Procedures

Culture Preparation

Hippocampal neurons were cultured conventionally (for some of the FM1-43 studies) or on microislands (for all other studies), as described previously (Furshpan et al., 1976; Bekkers and Stevens, 1991). Neurons were plated onto a feeder layer of astrocytes that had been laid down 1–7 days earlier (see Levison and McCarthy, 1991) and were used for experiments after 6–18 days in vitro. Older neurons, particularly those with large EPSCs, often failed to respond to application of phorbol and were not used for these studies.

Electrophysiology

Individual neurons constrained to a small island of permissive substrate will form synapses on themselves. Such connections are referred to as "autapses." All electrophysiology was performed on autaptic neurons.

Whole-cell voltage clamp recordings from autaptic neurons were carried out using an Axopatch 1B amplifier (Axon Instruments, Burlingame, CA). The extracellular solution contained 119 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 30 mM glucose, 20 mM HEPES, 1 μM glycine, and 0.1 mM picrotoxin (to block inhibitory GABAergic currents; Research Biochemicals International [RBI]). A low concentration of tetrodotoxin (TTX; 2 nM; RBI) was included to prevent spontaneous firing of neurons in all electrophysiological experiments, except those investigating the effects of high-frequency electrical stimulation on the rate of refilling.

Phorbols esters used included 0.1–10 μM PMA (phorbol 12-myristate 13-acetate), 10 mM PDA (phorbol 12,13-diacetate), and 0.1–0.5 μM PDBu (phorbol 12,13-dibutyrate); results with these phorbols were similar and were pooled together. Inactive phorbol 4-α-PMA (4-α-phorbol 12-myristate 13-acetate; 0.5–2 μM) was used as a negative control. All phorbols were purchased from RBI, made up as stock solutions in DMSO, and stored at −20°C. The final concentration of DMSO in diluted phorbol solutions was typically 0.1% or lower. Phorbol solutions were applied for 1–2 min using a puffer pipette controlled by a picospritzer or by bath application. PKC inhibitors H-7 (RBI), staurosporine (RBI), and bisindolylmaleimide I (Calbiochem) were bath applied for at least 30 min prior to application of phorbol. As described previously by Capogna et al. (1995), the effects of these inhibitors on cultured hippocampal neurons were quite variable but became less variable with longer incubation.

Recording pipettes of 2–3.5 MΩ were filled with 121.5 mM KGluconate, 17.5 mM KCl, 9 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, 0.2 mM EGTA, 2 mM MgATP, and 0.5 mM LiGTP. Some experiments were done using perforated patches; for these experiments amphotericin B (solubilized, Sigma) was included in the pipette solution. Access resistance was monitored, and only cells with stable access

resistance were included in the data analysis. The membrane potential was held at −60 mV, and EPSCs were evoked by triggering an unclamped sodium spike with a 0.5 ms depolarizing step. Data were acquired at a rate of 2 kHz. The size of the recorded EPSCs was calculated by integrating the evoked current to yield a charge value. Calculating charge value in this manner yields a direct measure of the amount of neurotransmitter released while minimizing the effects of cable distortion on currents generated far from the site of the recording electrode (the soma). For studies investigating the effects of active and inactive phorbols, only those cells whose EPSCs increased upon application of active phorbol were used for data analysis. For studies investigating the effects of PKC inhibitors, only cells from cultures that showed reliable, robust responses to phorbols in the absence of PKC inhibitors were used.

The amplitude and frequency of spontaneous mEPSCs were studied by recording continuously over hundreds of seconds in the presence of TTX (2 nM). Data for mEPSC analysis were acquired at a rate of 5 kHz. The peak amplitudes of the mEPSCs were measured off-line semiautomatically using an adjustable amplitude threshold. All deflections from baseline that were greater than the threshold were detected. Selected events were then visually examined, and any spurious events were manually rejected, while any missed events were flagged for inclusion in the mean amplitude and frequency calculations. Frequencies were calculated by dividing the total number of mEPSC events by the total time sampled.

Hypertonic Solution Application

The size of the readily releasable pool of vesicles was measured electrophysiologically by application of hypertonic solution (normal extracellular solution with 500 mM sucrose added) to an isolated autaptic neuron using a puffer pipette controlled by a picospritzer. A vacuum pipette was used to clear the hypertonic solution rapidly. The hypertonic solution was applied over the entire island on which the autaptic neuron was located, to ensure that the same population of synapses was activated every time the solution was applied. This solution evoked a large initial transient current that declined to a low steady-state level over about 3 s; hypertonic solution was applied for 4–5 s in order to deplete the readily releasable pool fully. Data were acquired at a rate of 200 Hz. With the broad area of application of hypertonic solution and relatively slow data acquisition rate used for this study, individual mEPSCs were poorly resolved and were only seen superimposed on the larger current. The size of the readily releasable pool was calculated by integrating the current evoked by the hypertonic solution to yield a charge value. To estimate the readily releasable pool size more accurately, we corrected the integral of the current by subtracting away the amount of steady-state exocytosis that occurred during the hypertonic solution flow (see Figure 1A). For experiments measuring the rate of refilling of the readily releasable pool, pairs of hypertonic challenges were applied, with a 1 min rest between the pairs. Rundown between the first and second challenge of a pair was corrected using the rate of rundown observed between consecutive first challenges.

FM1-43 Loading, Destaining, Imaging, and Analysis

The size of the readily releasable pool was also measured optically by labeling all the vesicles in the readily releasable pool with FM1-43. FM1-43 (10 μM; Molecular Probes) was bath applied while eliciting a train of 20–40 action potentials at a rate of 10–20 Hz using field electrodes, as described in Murthy and Stevens (1998). The FM1-43 solution was left on the cells for 50 s before perfusion with dye-free solution for at least 5 min. This protocol ensured that all vesicles in the readily releasable pool were exocytosed during the train of stimuli and had sufficient time to be stained and endocytosed before washing and removing any dye that had partitioned into the surface membrane. Optical imaging to monitor fluorescence was done with a Leica TCS confocal laser-scanning microscope. Images were acquired by computer using Leica TCS NT software and were analyzed using custom-written software (Murthy et al., 1997; Murthy and Stevens, 1998). Fluorescence was calculated in intensity units integrated over the pixels corresponding to a single synapse. The background fluorescence value was obtained at the end of the destaining stimulus protocol (1000 action potentials elicited at 10 Hz) and subtracted from all frames to give total releasable fluorescence. The

total releasable fluorescence was then divided by the value for single vesicle fluorescence to yield an estimate of the number of vesicles in the readily releasable pool (Murthy and Stevens, 1998). Fluorescence intensity per vesicle is remarkably constant from one preparation to the next, provided that dye concentration and laser power are kept constant. Because the dye concentration and laser power used for the FM1-43 studies reported here were identical to those used in Murthy and Stevens (1998), we used the values reported in that paper for our estimates of single vesicle fluorescence, after correcting for changes in the photomultiplier gain.

For all FM1-43 experiments, TTX was omitted from the extracellular solution (except where indicated during the poststimulus dye-loading phase), and 10 μM DNQX (RBI) and 50 μM D-APV (RBI) were included to prevent recurrent excitation and any potential activity-dependent changes in release probability. Phorbol (1 μM PMA) was bath applied 5 min after the end of the first destaining stimulus. Cells were incubated in the phorbol solution for 2 min before washout with the FM1-43 solution. The loading, imaging, and destaining protocol was then repeated to allow a direct comparison of the pool size (fluorescent intensity) at those sites that stained and destained both before and after phorbol application.

All data are presented as mean ± SEM.

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