Preferential potentiation of fast-releasing synaptic vesicles by cAMP at the calyx of Held

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We have studied the effects of cAMP on synaptic transmission at the calyx of Held and found that forskolin (an activator of adenylate cyclase) and 8-Br-cAMP (a membrane-permeable analog of cAMP) potentiated excitatory postsynaptic currents (EPSCs). Direct sampling of miniature EPSCs (mEPSCs) and nonstationary fluctuation analysis showed that mEPSCs were not modulated by cAMP, suggesting that the locus of modulation is presynaptic. Deconvolution was used to examine effects of cAMP on quantal-release rates. By using this method, it was shown recently that release probabilities of readily releasable vesicles are heterogeneous. Here, we show that cAMP selectively increases the number of vesicles with higher release probabilities, whereas a slow component of the EPSC, representing vesicles that fuse more slowly, is unchanged. cAMP increases the apparent Ca$^{2+}$ sensitivity for secretion, but this increase does not reflect an increase in release probability necessarily but rather an increase in the number of highly sensitive vesicles.

The synaptic strength is modified in an activity-dependent manner as well as by neuromodulators and second messengers (1–3). For elucidating the underlying mechanisms of these modulations, it is important to understand these various forms of synaptic plasticity in terms of changes in specific parameters of quantal release.

The calyx of Held allows simultaneous voltage-clamp recording from presynaptic and postsynaptic compartments (4, 5). By taking advantage of this technique and using a recently developed deconvolution method (6), we have shown that release probability ($P_r$) of readily releasable quanta is heterogeneous (7). Approximately one-half of the total number of quanta is released with a $P_r$ that is about 2- to 5-fold higher than that of the other half. Similar findings have been obtained at other synapses (8, 9). In this work, we investigated whether different populations of synaptic vesicles with distinct $P_r$ are modulated differentially. Such different modulation would also provide that the quanta with different $P_r$ are biochemically, not just electrophysiologically, distinct.

Based on this idea, we tried several pharmacological manipulations and found that drugs that elevate cAMP concentration lead to potentiation of excitatory postsynaptic current (EPSC) amplitude (see also ref. 10). In most preparations, cAMP potentiates quantal release presynaptically by activating protein kinase A (PKA; refs. 1 and 11–19). cAMP did not increase the amplitude of miniature EPSCs (mEPSCs) at the calyx of Held, suggesting a presynaptic origin of cAMP action. Analysis of quantal-release rates performed by using the deconvolution method (6) revealed that cAMP increased the apparent sensitivity of quanta to Ca$^{2+}$ influx, and it did so by selectively increasing the number of a subpopulation of readily releasable quanta with high $P_r$.

Methods

Slice Preparations and Recordings. Transverse slices of brainstem (150 to 200 μm in thickness) were cut from 8- to 10-day-old Wistar rats (4, 20). The recording chamber was perfused continuously with normal extracellular solution at a rate of 1 ml/s. Normal extracellular solution contained (in mM) 125 NaCl, 2.5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 25 glucose, 25 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 0.4 ascorbic acid, 3 myo-inositol, and 2 Na-pyruvate, bubbled with 95% O$_2$ and 5% CO$_2$ (pH 7.4, 320 milliosmolarity). A calyx terminal and the postsynaptic principal neuron were simultaneously whole-cell clamped at ~80 mV with an EPC-9/2 amplifier (HEKA-Electronics, Lambrecht/Pfalz, Germany). The presynaptic patch pipette (4–6 MΩ) was filled with a solution containing (in mM) 130–135 Cs-glucuronate, 20 tetrathylammonium chloride, 10 Hepes, 5 Na$_2$-phosphocreatine, 4 MgATP, 0.3 GTP, and 0.5 EGTA (pH 7.2 with CsOH; 310 milliosmolarity). EGTA (0.5 mM) was used to block facilitation, which masks the heterogeneity of $P_r$. In some experiments (Fig. 5), 0.05 mM BAPTA [1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetate] was used instead of EGTA. Then, 30–90% of the presynaptic series resistance (8–30 MΩ) was compensated. For the postsynaptic pipette (2–3.5 MΩ) solution, the concentration of EGTA was increased to 5 mM. The postsynaptic series resistance (3–10 MΩ) was compensated so that the uncompensated resistance was 2–3 MΩ. EPSC amplitudes were corrected off-line for the remaining deviation from the holding potential.

During recordings, 0.5 μM tetrodotoxin, 10 mM tetrathylammonium, 50 μM D-AP5, 100 μM cyclothiazide, and 1 mM kynurenic acid (Kyn) were added to the extracellular solution. Forskolin (50 μM; Calbiochem), 8-Br-cAMP (1 mM; sigma), and 3-isobutyl-1-methylxanthine (IBMX) (100 μM; Calbiochem) were added to the extracellular solution as indicated. When mEPSCs were sampled, bicuculline (10 μM) and strychnine (2 μM) were added to the extracellular solution, and Kyn was omitted from the extracellular solution. PKA inhibitors Rp-cAMP and KT-5720 were obtained from Calbiochem, and H-89 was from Sigma.

Deconvolution and Fluctuation Analysis. Release rates were estimated by using the deconvolution method adapted for the case of glutamatergic synapses (6). This method assumes that the total EPSC can be separated into a residual current because of residual glutamate in the synaptic cleft and a current component directly induced by quantal release. It determines residual glumarate by means of a simple diffusion model incorporated into the deconvolution algorithm. Because we had shown before that deconvolution is valid in the presence of cyclothiazide and Kyn (6), which block desensitization and saturation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, all experiments were carried out in their presence.

To estimate quantal sizes underlying evoked EPSCs, nonstationary fluctuation analysis was applied (6). A given protocol was repeated three to five times, and nonstationarities were elimi-
nated by subtracting consecutive traces from one another and by bandpass filtering. Variance was calculated and smoothed by a gliding window. The resulting estimate of variance was corrected for AMPA channel noise. Finally, we divided variance by the release rate, multiplied the result by a correction factor, which takes into account bandpass filtering of variance, and used this value as an index of mEPSC amplitudes.

Results
Potentiation of Evoked EPSCs by cAMP. The presynaptic Ca²⁺ current (Fig. 1A, I_pre, dotted line) was elicited by depolarization to +70 mV for 2 ms then repolarized to +40 mV for 30 ms (Fig. 1A, V_pre). This voltage protocol evoked a slow AMPA-receptor mediated EPSC (Fig. 1A, EPSC, dotted trace). We calculated the release rate (Fig. 1A, release rate, dotted trace), the peak of which was around 30 quanta ms⁻¹ (Fig. 1B). Extracellular application of forskolin (50 μM), an activator of adenylate cyclase, induced a marked potentiation of the EPSC (Fig. 1A, EPSC, solid trace) and of the release rate (Fig. 1A, release rate, solid trace) without changing the presynaptic Ca²⁺ current significantly. Here, the release rate was calculated assuming that forskolin did not modulate mEPSCs (see below). Potentiation of the release rate was more pronounced during the early period of the depolarizing pulse and less significant during the late period. The peak release rate was potentiated usually within 5–10 min after application of forskolin (Fig. 1B). Unfortunately, potentiation could not be documented for periods longer than 15 min because of run-down of transmitter release.

When the terminal was depolarized to values between +30 and +40 mV, the peak release rate at 5–10 min after forskolin was 200 ± 20% (mean ± SEM) of the control, whereas the Ca²⁺ current amplitude was 90 ± 4% of the control value (677 ± 74 pA; n = 4, Fig. 1 D and E). In an attempt to get larger potentiation, we coapplied forskolin and IBMX (100 μM), an inhibitor of phosphodiesterase (which hydrolyzes cAMP or cGMP). Although the release rate was potentiated slightly faster after application, we observed almost identical potentiation of the peak rate (206 ± 14%, n = 6; Fig. 1 E) without changing the presynaptic Ca²⁺ current (90 ± 3% of the control). 8-Br-cGMP (1 mM), a membrane-permeable analog of cGMP, potentiated neither the peak release rate (110 ± 9%, n = 4) nor the Ca²⁺ current (98 ± 5%). Therefore, it seems unlikely that cGMP potentiates EPSCs at the calyx of Held.

To confirm that potentiation was caused by cAMP, 1 mM 8-Br-cAMP was applied extracellularly. 8-Br-cAMP, a membrane-permeable analog of cAMP, potentiated the EPSC and the release rate (Fig. 1C, dotted traces; control, solid traces; in the presence of 8-Br-cAMP). Similar to forskolin, 8-Br-cAMP potentiated the release rate in the early period but much less in the later period of the depolarizing pulses (Fig. 1C). On average, the peak release rate and the Ca²⁺ current amplitude were 308 ± 57% and 98 ± 3% of control, respectively (n = 5; Fig. 1 D and E).
Effect of cAMP-Related Pharmacological Manipulations on mEPSCs.

To examine postsynaptic factors contributing to the potentiation, we examined whether mEPSCs were modulated by varying cAMP levels. Kyn was omitted from the extracellular solution to resolve mEPSCs reliably. Fig. 2A shows averaged mEPSCs before (dotted trace) and after (solid trace) application of 8-Br-cAMP, and mEPSC amplitudes and the time course of mEPSCs were similar. In addition, the cumulative mEPSC-amplitude distribution was identical (Fig. 2B). Mean mEPSC amplitudes were similar in control measurements (34.8 ± 2.9 pA, n = 12) and after the application of drugs (ratios to the control; forskolin, 1.08 ± 0.06; 8-Br-cAMP, 1.06 ± 0.03; forskolin + IBMX, 1.07 ± 0.03; n = 4 in each condition). The mEPSC decay, which was fitted with a double exponential [time constants of τ1 = 1.59 ± 0.14 ms (53%) and τ2 = 8.89 ± 0.92 ms in the control], did not change significantly [ratios to control, τ1 = 0.98 ± 0.07; proportion of fast component: 0.95 ± 0.08; τ2 = 0.95 ± 0.08; n = 12]. An increase in mEPSC frequency was observed only sometimes after the application, and we did not study it further.

Although the properties of spontaneous mEPSCs are not changed by cAMP-related drugs, it is possible that quantal events during massive exocytosis may be modulated by cAMP. Thus, we used nonstationary fluctuation analysis of evoked EPSCs to determine the properties of evoked mEPSCs. In Fig. 2C, we compared mEPSC amplitudes (under Kyn; ref. 6) measured before (○ with dotted line) and after (● with solid line) the forskolin application. In both cases, mEPSC amplitudes were the same (~11 pA). Fig. 2D shows the mean quantal amplitude obtained from fluctuation analysis before (left, 10.3 ± 1.2 pA) and after (right, 10.0 ± 1.2 pA; n = 2 from forskolin, n = 5 from forskolin + IBMX, n = 3 from 8-Br-cAMP) application of cAMP-related drugs. Estimated amplitudes are slightly smaller than previous estimates under similar conditions (~15 pA under cyclothiazide + Kyn; ref. 6). mEPSC amplitudes in the presence of drugs were 98 ± 3% of controls, and there were no differences among different types of drugs (forskolin, 98%; forskolin + IBMX, 101%; 8-Br-cAMP, 95%).

Effect of PKA Inhibitors on EPSCs.

In many preparations, quantal release is modulated by cAMP by the activation of PKA (14–18). To examine the role of PKA in cAMP-induced potentiation, we included 1 mM Rp-cAMP, a blocker of PKA, in the presynaptic patch pipette and applied the same protocol as shown in Fig. 1. Nevertheless, forskolin similarly potentiated the EPSC and the peak release rate (198 ± 9% of the control, n = 5) in a manner similar to control. Likewise, the intracellular application of 9 μM KT-5720, another blocker of PKA, failed to block potentiation (224 ± 7% relative to control before forskolin, n = 3). Furthermore, we preincubated the slice with 10 μM H-89, another PKA blocker, for 30–60 min and also applied 10 μM H-89 intracellularly, but forskolin still potentiated release (216 ± 19% relative to control before forskolin, n = 3). These results indicate that cAMP-induced potentiation described here is not mediated by PKA, which may also be the case in other preparations (21, 22).

cAMP-enhanced enhancement of synaptic transmission at the crayfish neuromuscular junction is mediated by modulation of presynaptic I0 (22). However, the extracellular application of 1 mM Cs+, which blocks I0, had no effect on forskolin-induced potentiation (n = 4).

cAMP Potentiates the Number of Fast-Releasing Synaptic Vesicles.

To examine the Ca2+ dependence of cAMP-induced potentiation, the presynaptic terminal was depolarized to three different potentials (Fig. 3A; black, +40 mV; red, +20 mV; blue, 0 mV) for 10 ms (test pulse). After an interval of 10 ms, the terminal was held at 0 mV for 20 ms to deplete the RRP of quanta (depleting pulse). Note the increase in both Ca2+ influx and release response during the test pulse when comparing the three conditions. An opposite relationship is observed during the depleting pulses, suggesting a limited number of quanta available for release. These three protocols were applied before (dotted traces) and after (solid traces) application of drugs (three cells each from forskolin, forskolin + IBMX, and 8-Br-cAMP). When the currents elicited by three different test potentials are compared, it can be seen that the release rates are potentiated more...
when presynaptic Ca\textsuperscript{2+} influx is smaller. As a result, cAMP shifts the Ca\textsuperscript{2+}-release relationship to the left (Fig. 3B; cAMP, solid line; control, right with dotted line). However, when the release rate at the end of the test pulse was plotted against the Ca\textsuperscript{2+} current amplitude, potentiation turned out to be small, particularly at large Ca\textsuperscript{2+} influx (Fig. 3B; cAMP, △ with solid line; control, Δ with dotted line). Remarkably, we observed only small (Fig. 3, when the test pulse was to +40 mV) or almost negligible potentiation (Fig. 3A, when the test pulse was to +20 and 0 mV) during depleting pulses. These findings suggest that cAMP does not simply increase the size of the RRP uniformly. In addition, cAMP does not simply increase \( P_r \) because in that case quanta should have been depleted faster.

To quantify the Ca\textsuperscript{2+} dependence of cAMP-induced potentiation, cumulative release during the test and the depleting pulses was calculated (Fig. 3A Bottom). Cumulative release during the test pulse was potentiated more when the Ca\textsuperscript{2+} influx was smaller. When the test pulse was +40 mV, cAMP potentiated the amount of release to 432 ± 71% of the control (Fig. 3C, \( n = 9 \)). Potentiation was less when the Ca\textsuperscript{2+} influx became larger (Fig. 3C, +20 mV; 201 ± 23% of the control, 0 mV; 136 ± 6%).

During the depleting pulse, cumulative release was increased only when the test pulse was to +40 mV (127 ± 8%; Fig. 3C). For the two other test potentials, cumulative release during the depleting pulse was found to be the same between before and after application of drugs (Fig. 3C), and time courses were almost identical (Fig. 3A). It has been shown that prolonged presynaptic depolarization to 0 mV evokes two components of quantal release with time constants of 2–3 ms and \( \sim 10 \) ms, respectively, when the presynaptic pipette contains 0.5 mM EGTA to block overlapping facilitation (7). Then, in the protocols of Fig. 3A, the fast component should be almost depleted...
during the test pulse to +20 or 0 mV, and release during the depleting pulse should reflect the slow component. Thus, the slow component of quantal release was not modulated by cAMP (see also Fig. 1 A and C). cAMP potentiated the total RRP size to 133 ± 6% of the control (1,926 ± 314 quanta; Fig. 3C).

For comparison, we increased \( P_r \) simply by eliciting more Ca\(^{2+}\) influx during the test pulse. We compared the amount of release in response to test pulses between +20 and 0 mV under control conditions. The amount of release during test pulses at 0 mV was 210 ± 25% of those at +20 mV, whereas the amount of release during depleting pulses decreased (61 ± 1%, Fig. 3D). The total pool size was almost the same (107 ± 2%) for both depolarization levels. Thus, cAMP-induced potentiation is different from simple up-regulation of \( P_r \) (Fig. 3D).

**Comparison Between cAMP-Induced Potentiation of Quantal Release and Facilitation Induced by Residual Ca\(^{2+}\).** It has been shown that facilitation (induced by presynaptic residual Ca\(^{2+}\)) increases \( P_r \) (23). To obtain more insights into cAMP-induced potentiation, we compared these two forms of synaptic modification.

To allow facilitation to occur, the presynaptic patch pipette was filled with a low Ca\(^{2+}\) buffering solution (0.05 mM BAPTA). The terminal was depolarized to +80 mV for 2 ms and was repolarized to +20 mV for 30 ms (Fig. 4A Left), which was long enough to deplete most of the RRP. Facilitation was induced by prepolarization of the terminal to 0 mV for 2–3 ms, which evoked the release of 287 ± 53 quanta, 17.9 ± 1.6% of the total RRP size \((n = 5)\). Prepulses shortened the time to peak release rate (Fig. 4B, when facilitated, 5.33 ± 0.58 ms; control, 9.20 ± 0.98 ms). Consistent with the idea that facilitation is caused by an increase in \( P_r \) only (23, 24), the release rate declined earlier, and the RRP size was unchanged (Fig. 4C, 103 ± 1%).

Forskolin also shortened the time to peak release rate (Fig. 4A Right), and the time to the peak release rate was 6.72 ± 0.05 ms (Fig. 4B). In contrast to facilitation, forskolin increased the amount of release in the early depolarization period without changing the late phase (Fig. 4A). As a result, the cumulative amount of release during the pulse increased to 129 ± 4% (Fig. 4C) of control.

**Discussion**

We have shown that forskolin and 8-Br-cAMP potentiate EPSCs at the calyx of Held synapse (Fig. 1). Individual quantal events were not modulated by cAMP (Fig. 2), suggesting a presynaptic locus of action. cAMP increased the apparent Ca\(^{2+}\) sensitivity of quantal release (Fig. 3), but potentiation was not simply caused by an increase in \( P_r \), which is the case in facilitation induced by residual Ca\(^{2+}\) (Fig. 4). Whereas facilitation did not change the total size of the RRP, cAMP selectively increased the number of a subset of quanta with high \( P_r \) (Figs. 3 and 4). It is unlikely that cAMP-induced potentiation is caused by an increase in presynaptic Ca\(^{2+}\) concentration, because presynaptic Ca\(^{2+}\) currents were not augmented (Fig. 1) and potentiation was observed in the presence of 0.5 mM EGTA, which is sufficient to chelate residual Ca\(^{2+}\) (25). In addition, depletion of releasable quanta should be faster if presynaptic Ca\(^{2+}\) concentration is elevated (Fig. 4), which was not observed in cAMP-induced potentiation.

Although specific inhibitors of PKA (Rp-cAMP, KT-5720, and H-89) effectively block the potentiation of transmitter release in other preparations (17, 21), they were ineffective at the calyx synapse. We do not know the exact reason why the inhibitors are ineffective. However, a recent finding by Beaumont and Zucker (22) indicates that at the neuromuscular junction, cAMP enhances quantal release through the up-regulation of \( I_h \) channels. This mechanism is unlikely to apply to the calyx of Held, because application of Cs\(^+\) had no effect on forskolin-induced potentiation. Interestingly, Beaumont and Zucker (22) observed a remaining PKA-independent potentiation of release that is not mediated by \( I_h \) channels. Thus, potentiation may partially share a common mechanism in both preparations. It will be interesting to determine whether some proteins involved in exocytosis possess cAMP-binding sites, such as recently identified cAMP sensitive guanine nucleotide exchange factors (26, 27).

In many preparations, cAMP-induced potentiation of quantal release is caused by an increase in \( P_r \) (14, 19). The present results are partially consistent with these findings, because the relationship between Ca\(^{2+}\) influx and release rate was shifted toward lower Ca\(^{2+}\) influxes (Fig. 3). However, potentiation is not caused simply by modulation of \( P_r \), which becomes evident from a comparison between cAMP-mediated potentiation and facilitation by residual Ca\(^{2+}\) (Fig. 4), which is generally attributed to an increase in \( P_r \) only (23).

The simplest explanation for the mechanism of potentiation is that potentiation is caused by a selective increase in the number of those synaptic vesicles (see also refs. 28 and 29) which have high \( P_r \). Large potentiation can be observed during smaller presynaptic Ca\(^{2+}\) influxes and especially at the very beginning of...
Selective modulation of different vesicle populations might also be the mechanism of other forms of synaptic modulation.

Augmentation induced by protein kinase C is suggested to result from an increase in the size of the RRP (32), but Yawo (33) suggested that the potentiation at the ciliary ganglion is caused instead by an increase in \( P_r \) (see also ref. 34). Perhaps both results may be reconciled if one assumes that the number of high \( P_r \) quanta is increased.

Under physiological conditions, the calyx of Held shows pronounced synaptic depression (20). cAMP is expected to potentiate the first few excitative postsynaptic potentials (EPSPs) during the train, but keeps subsequent EPSPs almost intact (35). The impact of this form of synaptic plasticity on computational aspects of auditory signal processing requires more detailed studies on neural coding (36, 37).

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