cAMP modulates intracellular Ca\(^{2+}\) sensitivity of fast-releasing synaptic vesicles at the calyx of Held synapse

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Running title: cAMP potentiates neurotransmitter release

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ABSTRACT

cAMP potentiates neurotransmitter release from the presynaptic terminal in many CNS synapses, but the underlying mechanisms remain unclear. Here, we addressed this issue quantitatively by performing double patch-clamp recordings from the pre- and postsynaptic compartments of the calyx of Held synapse in rat brain stem slices in combination with Ca$^{2+}$ uncaging. We found that elevation of cAMP increased intracellular Ca$^{2+}$ sensitivity for transmitter release, especially at lower Ca$^{2+}$ concentrations. The change in Ca$^{2+}$ sensitivity was limited to the fast-releasing synaptic vesicles, which could be released rapidly upon APs. cAMP did not affect the slowly releasing vesicles. Fit of the data using a simplified allosteric model indicated that cAMP increased the fusion ‘willingness’, thereby facilitating transmitter release. We suggest that synaptic vesicles have to be positionally primed to the release sites close to the Ca$^{2+}$ channel cluster for cAMP to modulate intracellular Ca$^{2+}$ sensitivity of transmitter release.

Key words: transmitter release; vesicle fusion; calcium sensitivity; release rate
INTRODUCTION

CAMP potentiates synaptic strength at many different types of synapses, a factor thought to underlie important physiological phenomena such as potentiation of synaptic efficacy (Bailey et al. 1996; Silva et al. 1998). Despite its importance, however, the underlying mechanisms remain incompletely understood. Modulation of synaptic strength arises from both pre- and postsynaptic mechanisms (Zucker and Regehr 2002).

For presynaptic effects, CAMP is suggested to potentiate transmitter release by increasing the probability of synaptic vesicle fusion and/or increasing the number of releasable vesicles, but these mechanisms are also not precisely determined. For example, it is unclear if CAMP modulates transmitter release probability by changing affinity for Ca\(^{2+}\), Ca\(^{2+}\) cooperativity, or the maximal rate of synaptic vesicle fusion (Neher and Sakaba 2008; Schneggenburger and Neher 2005). Biochemically, CAMP enhances transmitter release through activation of protein kinase A (PKA) in many preparations (Byrne and Kandel 1996; Capogna et al. 1995; Chavez-Noriega and Stevens 1994; Goy and Kravitz 1989; Kondo and Marty 1997; Llano and Gerschenfeld 1993; Trudeau et al. 1996; Weisskopf et al. 1994). In addition, PKA-independent actions of CAMP, which facilitate release of transmitters or hormones, have also been reported (Beaumont and Zucker 2000; Eliasson et al. 2003; Kashima et al. 2001; Ozaki et al. 2000).
The calyx of Held is a giant synapse in the auditory system that allows simultaneous whole-cell recordings of pre- and postsynaptic compartments (Barnes-Davies and Forsythe 1995; Forsythe 1994). At the calyx of Held synapse, the increase in cAMP level selectively increases the number of vesicles with a high release probability (fast-releasing synaptic vesicles), whereas the number of vesicles attributed to the slow component of release remains unchanged (Sakaba and Neher 2001b). In addition, cAMP facilitates transmitter release by increasing the release probability and the number of releasable vesicles equally (by 2- to 3-fold), an effect mediated through the cAMP–Epac pathway at the calyx synapse (Kaneko and Takahashi 2004).

These previous studies examined the synaptic responses evoked by presynaptic Ca\(^{2+}\) influx through Ca\(^{2+}\) channels. Thus, what remains unknown is if an increase in release probability arises from tighter coupling between Ca\(^{2+}\) channels and synaptic vesicles (Wadel et al. 2007) or from changes in the intrinsic Ca\(^{2+}\) sensitivity of synaptic vesicle fusion (Lou et al. 2005). In the present study, we examined the mechanism underlying cAMP-dependent synaptic potentiation using the Ca\(^{2+}\) uncaging technique (Bollmann et al. 2000; Schneggenburger and Neher 2000), which elevates the intracellular Ca\(^{2+}\) uniformly in the terminal and assays the Ca\(^{2+}\) dependence of transmitter release directly. Our results show that cAMP increases intracellular Ca\(^{2+}\)-sensitivity of fast-releasing synaptic vesicles for neurotransmitter release.
MATERIALS AND METHODS

Electrophysiology

Transverse brainstem slices (200 μm thick) were prepared from postnatal days 8 to 11 from Wistar rats (Forsythe 1994), according to the guidelines of German laws on animal protection. Slices were kept in an incubation chamber and continuously bubbled with 95% O2 and 5% CO2 at 37°C (at least 30 min to 1 h before the experiment). The standard extracellular solution contained (in mM) 125 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 25 glucose, 25 NaHCO3, 1.25 NaH2PO4, 0.4 L-ascorbic acid, 3 myo-inositol, and 2 Na-pyruvate, pH 7.3–7.4, 320 mOsm. In Fig. 1, 1 mM kynurenic acid (Kyn), 10 mM TEA-Cl, 1 μM TTX, 50 μM D-AP5, and 100 μM cyclothiazide (CTZ) were added to isolate presynaptic Ca2+ currents and postsynaptic AMPA receptor–mediated EPSCs.

For flash experiments, 2 mM γ-D-glutamylglycine (γ-DGG) was used instead of Kyn. CTZ and Kyn (or γ-DGG) were added to block desensitization and saturation of AMPA receptors and also to minimize voltage-clamp errors (Neher and Sakaba 2001a). The presynaptic pipette (4–7 MΩ) solution contained (in mM) 135 Cs-gluconate, 20 TEA-Cl, 10 Heps, 5 Na2-phosphocreatine, 4 MgATP, 0.3 NaGTP, and 0.2 EGTA, with pH adjusted to 7.2. The pre- and postsynaptic compartments were voltage-clamped at -80 mV with an EPC10/2 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany). The presynaptic series resistance (10–25 MΩ) was compensated by 30–90 %. The
postsynaptic pipette (3–4.5 MΩ) contained the same solution as the presynaptic pipette, except that EGTA was increased to 5 mM. For flash photolysis, the presynaptic patch pipette contained (in mM) 120 Cs-gluconate, 20 TEA-Cl, 20 Hepes, 0.5 MgCl2, 5 Na2ATP, 0.5 NaGTP, 1–2 DM-nitrophen, 0.85–1.7 CaCl2, and 0.2 fura-2FF or 0.1 fura-4F. The postsynaptic series resistance (3–8 MΩ) was compensated by the amplifier so that the remaining resistance was less than 3 MΩ. The remaining resistance was further compensated offline.

CTZ and D-AP5 were obtained from Tocris. DM-nitrophen, forskolin, and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Calbiochem. Fura-2FF and fura-4F were obtained from TefLabs. Other drugs were obtained from Sigma. CTZ was dissolved in DMSO, for a final concentration in the extracellular solution of 0.1%. Experiments were performed at room temperature.

Ca²⁺ uncaging and Ca²⁺ imaging

Ca²⁺ was uncaged from Ca²⁺-loaded DM-nitrophen using a flash lamp (Rapp, Germany), following the procedure of Schneggenburger and Neher (2000). We measured [Ca²⁺], with the ratiometric Ca²⁺ indicator dyes fura-2FF and fura-4F excited by UV light (at 350- and 380-nm wavelengths) using a monochromator (TILL, Photonics, Gräfelfing, Germany). Fluorescent images were obtained with an interline-transfer 640-×-480 pixel CCD (TILL Imago), controlled by the software TILLvisION.
Neutral density filters attenuated the flash intensity to produce $[\text{Ca}^{2+}]_i$ steps of different amplitudes. Images were analyzed offline in TILLvisION, followed by transfer of the resulting time series of fluorescence intensities of several pixels and the background fluorescence of a small area next to the calyx to Igor Pro 5.05A for additional analysis. 

$[\text{Ca}^{2+}]_i$ was calculated from the background-corrected fluorescence ratio at 350- and 380-nm wavelengths, after acquisition of the calibration constants. Calibration of $\text{Ca}^{2+}$ indicator dyes was performed in vitro; in vivo calibration has been shown to give similar calibration constants (Wadel et al. 2007).

Analysis

The deconvolution method. Quantal release rates were estimated by the deconvolution method adapted for the calyx of Held synapse (Neher and Sakaba 2001b). This method assumes that the total EPSC can be separated into a residual current caused by the delayed clearance of glutamate from the synaptic cleft and a current component evoked by quantal release events. In combination with fluctuation analysis, the deconvolution method has been shown to be valid in the presence of CTZ and Kyn (or $\gamma$-DGG), which block desensitization and possible saturation of the postsynaptic AMPA receptors (Neher and Sakaba 2001a; b; Wadel et al. 2007). Quantal release rates, as determined by deconvolution, were integrated to obtain the cumulative release. For calculating release
rates per vesicle, the transmitter release rates were divided by the total size of the releasable vesicle pool.

Estimation of release probability during a train of AP-like stimuli. The method was similar to that developed by Schneggenburger et al. (1999) and Hosoi et al. (2007). During a 100-Hz train, the postsynaptic responses are depressed and reach a steady-state at the calyx of Held synapse. It was assumed that replenishment of new synaptic vesicles maintains the steady-state level. Based on this assumption, a differential equation was constructed and solved numerically by varying the initial size of the releasable pool of vesicles and the replenishment time constant until the rate of vesicle replenishment could fully explain the release rates during the steady-state (late in the AP train). Then, the release probability was calculated for each pulse by dividing the amount of release during an AP-like stimulation by the size of the remaining vesicle pool. At the calyx of Held synapse, both fast- and slowly releasing components of release have been reported (Sakaba 2006). Because the fast- and slowly releasing vesicles could not be separated precisely under physiological conditions (Hosoi et al., 2008 for detailed procedure for separation), the releasable pool of vesicles represents the summation of the two components. The data are expressed as mean ± SEM. Two-tailed Student’s t tests were done to determine significant differences ($P < 0.05$).
RESULTS

Potentiation of EPSCs by forskolin

Bath application of forskolin, an adenylyl cyclase activator, can potentiate EPSCs evoked by a step depolarization of the presynaptic terminal or by fiber stimulation (Kaneko and Takahashi 2004; Sakaba and Neher 2001b). To examine how cAMP modulates the probability of synaptic vesicle fusion, we made whole-cell recordings from both the pre- and postsynaptic compartments of the calyx of Held synapse. The EGTA concentration in the presynaptic patch pipette solution was 0.2 mM to mimic the physiological intracellular Ca\(^{2+}\) buffering system (Borst and Sakmann 1996; Müller et al. 2007). To observe the cAMP-induced potentiation of transmitter release rapidly, we also added IBMX, an inhibitor of phosphodiesterase that hydrolyzes cAMP or cGMP, to the extracellular solution in all experiments. The presynaptic Ca\(^{2+}\) currents and EPSCs were evoked by a 100-Hz train of action potential (AP)-like stimuli (depolarization to +40 mV for 0.4 to 1 ms, and then ramp to -80 mV for 0.8 ms), followed by a step-depolarizing pulse (depolarization to 70 mV for 2 ms followed by repolarization to 0 mV for 50 ms) to deplete the remaining vesicle pool. The duration of each AP-like stimulus was adjusted to achieve a relatively small amplitude for the first EPSC, so that we could observe the large potentiation effect.
Extracellular application of forskolin and IBMX induced a marked potentiation of the EPSCs without changing the amplitudes of presynaptic Ca\(^{2+}\) currents significantly (Fig. 1A). We calculated the release rates from the EPSCs by the deconvolution method (Neher and Sakaba 2001a; b), assuming that forskolin + IBMX did not modulate the miniature EPSC (mEPSC) amplitudes, as verified in previous studies (Kaneko and Takahashi 2004; Sakaba and Neher 2001b). The peak release rate during the first AP was 930 quanta ms\(^{-1}\) and the cumulative release was 10,500 quanta under control conditions (at the end of the protocol) (Fig. 1A). After application of forskolin + IBMX, the peak release rate of the first EPSC was increased to 2360 quanta ms\(^{-1}\), and the cumulative release was increased to 13,390 quanta. A step-depolarizing pulse following AP-like stimuli evoked only slow release, indicating that the fast-releasing synaptic vesicles responsible for AP-triggered release had been mostly depleted in our AP-like stimuli (Sakaba 2006). Depletion of the vesicle pool is important for calculating release probability (see below).

On average, the amount of presynaptic Ca\(^{2+}\) influx was identical between control and forskolin + IBMX -treated conditions (1.00 ± 0.04-fold, \(P = 0.95, n = 9\)). The amplitude of the first EPSC in an AP train was increased more than 2-fold (2.21 ± 0.36-fold, \(P < 0.01, n = 9\)). Potentiation was observed especially during the initial few stimuli in the AP train. The peak release rate of the first stimulus also was increased 2-fold (2.1 ± 0.32-fold, \(P < 0.01, n = 9\)). The size of the releasable vesicle pool was calculated from
the cumulative release, as described in the method. The pool size was variable among
cell pairs under control conditions (1600 to 13,500 vesicles; mean = 6200 ± 1680, n =
9); this number includes both the fast- and the slowly releasing vesicles at the calyx of
Held synapse (Sakaba and Neher 2001c). On average, forskolin increased the total size
of releasable vesicles by 20 % (1.20 ± 0.04, P < 0.001, n = 9) (Fig. 1B).

We divided the number of released vesicles during each AP by the pool size to obtain
the release probability. Forskolin increased the release probability of the first six stimuli
in a stimulus train, and the potentiation was largest during the first stimulation. On
average, the release probability in response to the first stimulus increased 1.84 ± 0.22-
fold (P < 0.01, n = 9) (Fig. 1B). Thus, these results suggest that forskolin + IBMX
increases both the release probability (by 80 %) and the number of readily releasable
vesicles (by 20 %) during a train of AP-like stimuli at the calyx of Held synapse.

Increase in the release probability could be due to tighter coupling between Ca\textsuperscript{2+}
channels and synaptic vesicles (Wadel et al. 2007) or increased Ca\textsuperscript{2+} sensitivity of
synaptic vesicles for fusion. In the following experiments, we measured the release
probability directly using flash photolysis of caged Ca\textsuperscript{2+} (Lou et al. 2005;
Schneggenburger and Neher 2000).

cAMP augments transmitter release induced by presynaptic Ca\textsuperscript{2+} uncaging
We performed simultaneous pre- and postsynaptic whole-cell recordings combined with presynaptic Ca\textsuperscript{2+} uncaging to elevate the intracellular Ca\textsuperscript{2+} concentration uniformly (Fig. 2). [Ca\textsuperscript{2+}]\textsubscript{i} was monitored using the Ca\textsuperscript{2+} indicator dye fura-2FF. 60 ms after flash photolysis of caged Ca\textsuperscript{2+}, a step-depolarizing pulse was applied to deplete the remaining releasable vesicles. We used different flash intensities to elevate [Ca\textsuperscript{2+}]\textsubscript{i} to different levels. Fig. 2 shows an example applying a flash of intermediate intensity, which elevated [Ca\textsuperscript{2+}]\textsubscript{i} to 5.5 µM under control conditions and 4.8 µM after the application of forskolin + IBMX. Although the peak [Ca\textsuperscript{2+}]\textsubscript{i} level was slightly lower after the application of forskolin + IBMX, the peak flash-evoked EPSC amplitude increased from 2.5 nA to 4.7 nA. The peak release rate increased from 74 vesicles ms\textsuperscript{-1} to 150 vesicles ms\textsuperscript{-1}. The size of the releasable pool of vesicles (at the end of a depleting pulse) also increased by 16 % in this cell pair (21 ± 7 % on average when the post-flash [Ca\textsuperscript{2+}]\textsubscript{i} was between 3 to 6 µM; n = 5), similar to the result obtained from a train of AP-like stimuli. Then, we applied different intensities of flash stimuli. Interestingly, when [Ca\textsuperscript{2+}]\textsubscript{i} was elevated to around 10 µM, there was only a small potentiation of EPSC amplitudes (Fig. 3A). A subsequent depolarizing pulse evoked almost no EPSC, indicating depletion of the vesicle pool. The peak release rate and cumulative release increased only slightly when the control trace and the one following forskolin application were compared. The vesicle pool size was increased 1.13 ± 0.05-fold (n = 4).
Fig. 3B summarizes the uncaging experiments showing transmitter release rate per vesicle as a function of $[\text{Ca}^{2+}]_\text{i}$, both for the control conditions and after application of forskolin + IBMX. Release rates per vesicle were calculated by dividing the peak release rate by the total size of the releasable vesicles (Wadel et al. 2007). We applied linear regression to the whole data set after logarithmic transformation. The control data (Fig. 3B) give a slope of $4.43 \pm 0.37$. Conversely, the data obtained in the presence of forskolin + IBMX give a slope of $3.07 \pm 0.27$, which indicates that forskolin reduces the apparent cooperativity with which $[\text{Ca}^{2+}]_\text{i}$ induces vesicle fusion.

Forskolin also affected the synaptic delay. We plotted the synaptic delay (the time between the triggering of the flash and a level crossing of five released quanta in the cumulative release trace) as a function of $[\text{Ca}^{2+}]_\text{i}$. The synaptic delay was shortened after the application of forskolin + IBMX for $[\text{Ca}^{2+}]_\text{i}$ steps below 7 $\mu$M, indicating that cAMP accelerated transmitter release kinetics. We concluded that when the concentration of cAMP was elevated in the presynaptic terminal, the intracellular $\text{Ca}^{2+}$ sensitivity of vesicle fusion increased, especially at lower $\text{Ca}^{2+}$ concentrations in the low micromolar range.

**cAMP increases transmitter release rates around basal $[\text{Ca}^{2+}]_\text{i}$**

The flash experiments showed that forskolin increased the intracellular $\text{Ca}^{2+}$ sensitivity of vesicle fusion in response to submaximal stimuli of $[\text{Ca}^{2+}]_\text{i}$. We next
examined if forskolin affected transmitter release rates at lower \([\text{Ca}^{2+}]_i\), close to the basal level. To address this issue, we preloaded calyces with EGTA/Ca\(^{2+}\)-containing solution to clamp \([\text{Ca}^{2+}]_i\) below 500 nM. The presynaptic \([\text{Ca}^{2+}]_i\) was monitored with the high-affinity \text{Ca}^{2+}\ indicator fura-4F. Spontaneous mEPSCs were recorded during the initial 2–5 min after the formation of whole-cell configuration for control, and forskolin + IBMX was applied subsequently. In Fig. 4A, \([\text{Ca}^{2+}]_i\) was clamped around 130 nM, and forskolin + IBMX increased the frequency of spontaneous mEPSCs from 2.65 Hz to 10.3 Hz without a significant change in \([\text{Ca}^{2+}]_i\). When the clamped presynaptic \([\text{Ca}^{2+}]_i\) was increased, the mEPSC frequency also increased under control conditions but the \text{Ca}^{2+}\-dependence was shallow in the low \text{Ca}^{2+}\ range (see Fig. 6, for summary). Across this experiment, forskolin + IBMX increased mEPSC frequency 4.2 ± 0.4-fold when the \([\text{Ca}^{2+}]_i\) was below 500 nM. The potentiation of transmitter release was more prominent than that observed during a train of AP-like stimuli or in flash experiments, which covered the \([\text{Ca}^{2+}]_i\) levels > 2 μM.

It is difficult to clamp \([\text{Ca}^{2+}]_i\) continuously around 1 μM using an EGTA/Ca\(^{2+}\) mixture because the relatively high rate of secretion causes rapid rundown of release, most likely arising from depletion of the recycling pool of vesicles. Therefore, we used flash photolysis with DM-nitrophen but attenuated flash intensity with a neutral density filter to produce a small increment of \([\text{Ca}^{2+}]_i\), (600 nM–2 μM). At the same time, we used the UV light from the monochromator to keep \([\text{Ca}^{2+}]_i\) stable for a few seconds,
which also served for fluorescent measurement of $[\text{Ca}^{2+}]_{i}$. $[\text{Ca}^{2+}]_{i}$ was monitored with the Ca$^{2+}$ indicator fura-4F. The mEPSC frequency during this stable period was measured. Fig. 4B shows a typical example of such an experiment. Flash photolysis elevated $[\text{Ca}^{2+}]_{i}$ to around 900 nM. We counted the number of mEPSCs for the last 4 s in the trace, where the $[\text{Ca}^{2+}]_{i}$ was relatively stable, and found that forskolin + IBMX markedly increased the mEPSC frequency by 3.75-fold in this example.

Selective modulation of the fast-releasing synaptic vesicles by cAMP at the calyx of Held synapse

At the calyx of Held synapse, two components of transmitter release have been observed during a prolonged depolarization (Sakaba and Neher 2001a), which have the release time constants of 2–3 ms and 10–30 ms, respectively. The two sets of vesicles arise from a difference in the proximity to the Ca$^{2+}$ channel clusters (Wadel et al. 2007). It has been shown that the fast-releasing vesicles, but not slowly releasing vesicles, are sensitive to cAMP at the calyx of Held (Sakaba and Neher 2001b). However, phorbol esters, which have an effect similar to that of cAMP described here, seem to potentiate all the vesicles equally (Lou et al. 2005). It is possible that cAMP accelerates the release time constant of all of the vesicles and results in faster vesicle pool depletion, but the pool depletion was compensated by the increase in the number of releasable vesicles.
a result, the late component of transmitter release appears to be unchanged, but that does not mean that cAMP does not modulate slowly releasing vesicles.

To examine whether cAMP does modulate intracellular Ca\textsuperscript{2+} sensitivity of the slowly releasing vesicles, we isolated the slowly releasing vesicles by a pre-depolarizing pulse (0 mV for 10–15 ms following a pre-pulse to +70 mV for 2 ms), which depleted the fast-releasing vesicles (Fig. 5A). After the pre-pulse, the test pulse was applied to release the slowly releasing synaptic vesicles. Calmodulin inhibitory peptide (20 µM) was included to prevent recovery of the fast-releasing vesicles between the two pulses (Sakaba and Neher 2001a; Wadel et al. 2007). As Fig. 5A shows, the following test pulse evoked a more slowly rising EPSC. Accordingly, cumulative release also showed a slow increase, indicating that only slowly releasing vesicles were released during the test pulse. Forskolin + IBMX potentiated the EPSC during the pre-pulse, but not the one during the test pulse, confirming previous results (Sakaba and Neher 2001b).

In addition to applying a test pulse, we applied flash photolysis to assay the intracellular Ca\textsuperscript{2+} sensitivity of the slowly releasing vesicles (shown in Fig. 5B). Flash intensity was adjusted so that [Ca\textsuperscript{2+}]\textsubscript{i} was increased to 3–6 µM, at which level the cAMP-induced potentiation of release was relatively large with a stand-alone flash photolysis (Fig. 2). As Fig. 5B shows, forskolin + IBMX potentiated the pre-pulse EPSCs but the flash-evoked EPSCs were not modulated significantly (Fig. 5B), indicating little modulation of the slowly releasing vesicles by cAMP. Fig. 5C
summarizes the relationship between the release rate per vesicle and \([\text{Ca}^{2+}]\). Under control conditions, release rates per vesicle of the slowly releasing vesicles were the same as the fast-releasing component (compare Figs. 3 and 5), indicating that the two components were equally release-competent when \([\text{Ca}^{2+}]\) was elevated uniformly. Unlike the outcome depicted in Fig. 3, however, forskolin +IBMX did not potentiate the release rate per vesicle. The results indicate that only the intracellular \(\text{Ca}^{2+}\) sensitivity of the fast-releasing vesicles was modulated.

Modelling the \(\text{Ca}^{2+}\)-dependent synaptic vesicle fusion with a simplified allosteric model

In Fig. 6A, the data obtained from \([\text{Ca}^{2+}]\)-clamp experiments, small flashes, and normal flashes were pooled together and the peak release rate per vesicle was plotted against \([\text{Ca}^{2+}]\). Note that release rates per vesicle were calculated by dividing the peak release rate by the size of the total release-ready vesicle pool, which includes both the fast- and the slowly releasing vesicles. This procedure is the same as that used previously (Wadel et al. 2007). If the slowly releasing vesicles were excluded, the release rates per vesicle would be 2-fold higher, but the overall conclusions described below remain the same.

We found that the \(\text{Ca}^{2+}\) cooperativity of vesicle fusion gradually decreased at lower \([\text{Ca}^{2+}]\), under control conditions (Lou et al. 2005). This feature cannot be described by a
sequential Ca\(^{2+}\)-binding model, which assumes that Ca\(^{2+}\) binds to the Ca\(^{2+}\) sensor of synaptic vesicles sequentially and that vesicle fusion occurs exclusively from the fully occupied state (Bollmann et al. 2000; Schneggenburger and Neher 2000). This sequential model predicts a release rate of 0 at zero [Ca\(^{2+}\)]\(_i\), which is not the case for the experimental data (Fig. 6A). Alternatively, we fitted the data with a simplified allosteric model (Lou et al. 2005), in which the rate \(l_+\) allows low rates of vesicle fusion in the absence of bound Ca\(^{2+}\) and increasingly higher rates of vesicle fusion are attained when the Ca\(^{2+}\) sensor is more completely occupied by Ca\(^{2+}\) (Lou et al. 2005) (Fig. 6B). For control data, the parameters were determined as follows: \(k_{on} = 1.5 \times 10^8\) M\(^{-1}\)s\(^{-1}\), \(k_{off} = 6000\) s\(^{-1}\), \(l_+ = 3 \times 10^4\) s\(^{-1}\), \(f = 30\), \(b = 0.5\). The forskolin-treated data could be fitted by changing \(l_+\) to 2 \times 10^3\) s\(^{-1}\), without changing other parameters. According to the model, forskolin + IBMX increases the basal fusion ‘willingness’, similar to the effect of phorbol esters at the calyx of Held synapse (Lou et al. 2005). The increase in \(l_+\) predicts enhanced fusion rates not only at basal [Ca\(^{2+}\)]\(_i\) but also at intermediate [Ca\(^{2+}\)]\(_i\) (2–7 μM). Recently, a dual-Ca\(^{2+}\)-sensor model of synaptic vesicle fusion has been proposed, based on the results obtained from synaptotagmin 2 KO mice (Sun et al. 2007). This model postulates that synaptotagmin 2 is the fast Ca\(^{2+}\) sensor, which is responsible for synchronous release while another undefined slow Ca\(^{2+}\) sensor is responsible for asynchronous release. In addition, at zero [Ca\(^{2+}\)]\(_i\), vesicle release takes place at a very low rate with a rate constant (\(\gamma_1\) in supplementary Fig 1). In a dual-Ca\(^{2+}\) sensor model,
three (fast, slow, and basal) modes of release can be selected depending on the Ca\(^{2+}\) level in a given synaptic vesicle. In order to test which Ca\(^{2+}\) sensor is affected by cAMP, we fitted both control and forskolin-treated data with a dual-Ca\(^{2+}\)-sensor model (Supplementary Fig 1). We found this model fitted well with the data, similarly to the allosteric model. According to the model, forskolin + IBMX changed the Ca\(^{2+}\)-unbinding rate of all the sensors.

Finally we examined whether the cAMP-induced potentiation during an AP-like stimuli (Fig 1) could be predicted by the release model. In Fig. 7A, an AP-like stimulus was generated by the ramp stimulation protocol, which depolarized the presynaptic terminal to +40 mV for a short period and then repolarized back to -80 mV. In response to the ramp, presynaptic Ca\(^{2+}\) current was elicited, which could be fit by a Gaussian function with a half width of 332 ± 12 μs (n = 5 cells). In response to the presynaptic Ca\(^{2+}\) influx, the EPSC was evoked, and transmitter release rates were calculated from the deconvolution method. The EPSCs were potentiated approximately 2-fold after application of forskolin + IBMX (Fig. 7B for average time course of release from 5 cell pairs), whereas presynaptic Ca\(^{2+}\) current amplitudes stayed the same or slightly decreased (0.92 ± 0.04). In Fig. 7B, we examined whether the allosteric model could explain the cAMP-induced potentiation of transmitter release. The parameters of the model were the same as those determined from the dose-response curve of Fig. 6. First, the time course of transmitter release under the control condition was simulated by
varying the Ca\(^{2+}\) waveform until it fitted the data well. It turned out that a Gaussian waveform with a half width of 450 \(\mu s\) and peak amplitude of 10 \(\mu M\) could simulate the time course of transmitter release evoked by the AP-like stimulus under control condition. Gaussian width was slightly larger than that of the presynaptic Ca\(^{2+}\) current, possibly reflecting diffusion of Ca\(^{2+}\) from Ca\(^{2+}\) channel cluster. Nevertheless, the peak amplitude of [Ca\(^{2+}\)]\(i\) was comparable to that of the previous studies (Bollmann et al., 2000; Schneggenburger and Neher, 2000). The same Ca\(^{2+}\) waveform (but reducing the peak Ca\(^{2+}\) amplitude to 92 \%, according to the reduction in Ca\(^{2+}\) currents) was used to simulate the time course of release under forskolin + IBMX. In addition, we also took into account that the RRP size was increased by 20 \% after addition of forskolin + IBMX. The simulated time course of transmitter release matched quite well with the time course of release obtained experimentally, indicating that changes in the transmitter release kinetics and the RRP size could explain the cAMP-induced potentiation of transmitter release quantitatively. Ca\(^{2+}\) is not equilibrated with the Ca\(^{2+}\) sensors and Ca\(^{2+}\) -binding steps are not fully occupied during an AP, because Ca\(^{2+}\) rises and falls very rapidly. In contrast, flash photolysis elicited a Ca\(^{2+}\) step for longer period, under which Ca\(^{2+}\) is equilibrated with the sensors. Therefore, cAMP-induced potentiation during an AP cannot be simply predicted from the dose-response curve of Fig 3. In simulation, forskolin + IBMX potentiated transmitter release during an AP-like stimulation more effectively compared with the one evoked by flash photolysis even
though the peak Ca\textsuperscript{2+} amplitudes were the same. In supplementary Fig. 2, we did the
same type of simulation using the 2-sensor model, which could explain the experimental
results similarly.
At the calyx of Held synapse, cAMP potentiates neurotransmitter release (Kaneko and Takahashi 2004; Sakaba and Neher 2001b). In the present study, we examined how cAMP modulates the intracellular Ca\(^{2+}\) sensitivity of transmitter release. The Ca\(^{2+}\) perfusion and the Ca\(^{2+}\) uncaging techniques were used to explore the intrinsic Ca\(^{2+}\) sensitivity of synaptic vesicle fusion. Forskolin + IBMX increases the Ca\(^{2+}\) sensitivity of vesicle fusion predominantly at lower [Ca\(^{2+}\)]\(_i\). This is similar to the effect of phorbol esters demonstrated by Lou et al. (Lou et al. 2005); however, the potentiation was limited in the current study to the fast-releasing synaptic vesicles, and cAMP did not affect the slowly releasing vesicles.

**Enhancement of release probability by cAMP at the calyx of Held synapse**

The cAMP-induced potentiation of transmitter release could be the result of an increase in the release probability, the number of releasable vesicles, or both. In the cerebellar parallel-fibre–Purkinje cell synapse (Chen and Regehr 1997) and cultured excitatory and inhibitory hippocampal synapses (Gekel and Neher 2008), most of the enhancement arises from increase in the release probability, although small changes in the size of the vesicle pool could not be excluded. At the calyx of Held synapse, cAMP strongly potentiated transmitter release when [Ca\(^{2+}\)]\(_o\) was reduced (Kaneko and...
Takahashi 2004). In their study, cAMP increased both the release probability and the number of releasable vesicles equally. At the same preparation, Sakaba and Neher found that cAMP increased the number of fast-releasing vesicles by ~25% (Sakaba and Neher 2001b). In the present study, the flash photolysis results indicate that an increase in intracellular Ca$^{2+}$ sensitivity largely mediates the potentiation, similar to the case of phorbol esters (Lou et al. 2005). cAMP did not change the coupling between Ca$^{2+}$ channels and synaptic vesicles, which potentially accelerate the transmitter release kinetics. Furthermore, we have shown that cAMP alters intracellular Ca$^{2+}$ sensitivity of fast-releasing vesicles for transmitter release, but not of slowly releasing vesicles. Fast- and slowly releasing vesicles are distinguished by release kinetics during a prolonged depolarization (time constants of 2–3 ms and 10–30 ms, respectively; Wu and Borst, 1999; Sakaba and Neher, 2001a). The fast-releasing vesicles are proposed to be located close to the Ca$^{2+}$ channel cluster, whereas the slowly releasing vesicles are located further away from Ca$^{2+}$ channels (Wadel et al. 2007). For the phorbol ester effect, the differential influence on the two sets of vesicles has not been tested. A small increase in the fast-releasing vesicles is possibly related to the cAMP-dependent of vesicle replenishment at the calyx of Held (Sakaba and Neher 2003), and enhancement of vesicle replenishment rate may increase the vesicle number at rest.

Wölfel et al. showed that flash photolysis evoked two kinetically distinct components of release (Wölfel et al. 2007). They postulated that the difference comes from the speed
of ‘willingness’ for fusion (or Ca$^{2+}$-independent secretion step following the Ca$^{2+}$-dependent steps) for the two vesicle pools, which leads to the different release kinetics for the two components of release observed during a depolarizing pulse, and which they see also in response to spatially uniform [Ca$^{2+}$]$_i$ steps. In contrast, Wadel et al. (2007) found little difference (2-fold at most) in Ca$^{2+}$ sensitivity of the two components of release observed during a depolarizing pulse. They found that the two components have similar intrinsic kinetics in response to a spatially uniform [Ca$^{2+}$]$_i$ step, leading them to conclude that the two components of release seen to depolarizing pulses arise from the different distances of two classes of vesicles from Ca$^{2+}$ channels – those that are attached to a channel and those that are not. Comparison of the Ca$^{2+}$ sensitivity between fast- and slowly-releasing vesicles in this study (comparison between Fig 3 and 5) seems to support Wadel et al (2007). Alternatively, the slow component of Wölfel et al (2007) observed under flash photolysis experiment may not be released in the protocol used in this study. Recently, it has been reported that biochemical heterogeneity within the fast-releasing synaptic vesicles existed at the calyx synapse (Müller et al. 2010), and Wadel et al. (2007) also noted a relatively small (2-fold) difference in the Ca$^{2+}$ sensitivity among releasable synaptic vesicles. It is possible that the cAMP action may be related to the biochemical heterogeneity.

cAMP changes only the intracellular Ca$^{2+}$ sensitivity of the fast-releasing vesicles, also changes the frequency of spontaneous release near resting Ca$^{2+}$. Therefore, it is
reasonable to postulate that the fast-releasing vesicles, at least in part, mediate spontaneous release. This is consistent with (Xu et al. 2009), suggesting that spontaneous release does not necessarily require a Ca^{2+}-sensing mechanism different from evoked transmission under control condition. However, we cannot entirely exclude the possibility that part of spontaneous release may be mediated by another Ca^{2+} sensor and/or that the mode of transmitter release (evoked or spontaneous) is chosen in a given synaptic vesicle depending on the Ca^{2+} level (Sun et al., 2007).

The mechanisms underlying the intracellular Ca^{2+} sensitivity of the fast-releasing synaptic vesicles

We used Ca^{2+} uncaging to uniformly elevate [Ca^{2+}]_i in the presynaptic terminal to study the intrinsic Ca^{2+} sensitivity of vesicle fusion under modulation by second messengers. In the range of 2–14 μM [Ca^{2+}]_i, the cooperativity of Ca^{2+} for transmitter release was high under control conditions (around 4), consistent with previous reports (Lou et al. 2005; Schneggenburger and Neher 2000). cAMP increased the Ca^{2+} sensitivity of vesicle fusion, but this enhancement becomes less prominent with an increase in [Ca^{2+}]. Accordingly, the Ca^{2+} cooperativity was reduced from 4 to 3. The mean mEPSC frequency under basal Ca^{2+} conditions was increased 4–5-fold in the current work, and all data sets were fitted by a simplified allosteric Ca^{2+}-binding model (Lou et al. 2005). This model adds the rate constant $l_s$ to the conventional sequential
Ca\textsuperscript{2+}-binding model (Heinemann et al. 1994; Lando and Zucker 1994; Schneggenburger and Neher 2000), allowing low rates of vesicle fusion to occur in the absence of bound Ca\textsuperscript{2+} at the Ca\textsuperscript{2+} sensor. In this model, increasingly higher rates of vesicle fusion can be attained when the Ca\textsuperscript{2+} sensor is more completely occupied by Ca\textsuperscript{2+}. Compared with control data, application of forskolin increased the vesicle fusion rate constant \( l \) \( \pm 6.7 \)-fold. According to this view, the change in Ca\textsuperscript{2+} cooperativity is not necessarily mediated by changes in the Ca\textsuperscript{2+} sensing mechanism itself. Rather ‘willingness’ of fusion is changed. This outcome is almost the same effect as seen with phorbol esters, which stimulate the presynaptic protein kinase C/munc-13 signalling cascades (Hori et al. 1999; Rhee et al. 2002; Silinsky and Searl 2003) and also increase \( l \), 5-fold (Lou et al. 2005). cAMP and protein kinase C may share a similar mechanism of action on transmitter release by perhaps having the same downstream target (but, see below).

Application of the Epac agonist 8-pCPT-2’-O-Me-cAMP (ESCA1) augmented a subsequent enhancement of evoked EPSC amplitudes by phorbol esters in cultured mouse autaptic neurons from the hippocampus, and this effect was maximal when ESCA1 application preceded the PDBu application by 3 min, indicating a downstream effect of cAMP in Epac-to-PKC signalling (Gekel and Neher 2008). Kaneko and Takahashi (2004) showed, however, that the forskolin- and phorbol ester effects were additive, arguing against a common target. The molecular mechanism of cAMP action remains to be elucidated.
It is possible that models of synaptic vesicle fusion other than an allosteric model may explain the results. For example, the two-sensor model proposed by Sun et al. (2007) can be considered. According to this model, fast and slow Ca\(^{2+}\) sensors are responsible for fast transmitter release and spontaneous release near resting Ca\(^{2+}\), respectively. Then, one has to postulate that both sensors will be modulated by cAMP. Because spontaneous release near basal Ca\(^{2+}\) is strongly modulated by cAMP, one also has to postulate that cAMP would strongly shift the Ca\(^{2+}\) affinity of the slow sensor (specifically, Ca\(^{2+}\) unbinding rate of the slow sensor). The changes in the apparent Ca\(^{2+}\)-cooperativity (Fig 3) can be explained by stronger modulation of the slow sensor over the fast one. Therefore, irrespective of an allosteric or a two-sensor model, cAMP does not have to modulate the Ca\(^{2+}\)-sensing mechanism directly, although the possibility of direct modulation of Ca\(^{2+}\) sensors cannot be entirely excluded. It is important to note that the two-sensor model cannot explain cAMP-induced potentiation fully: During a train of APs, the model predicts that synaptic delays get progressively shorter under forskolin + IBMX, which is inconsistent with the common observation that synaptic delays are relatively the same. On the other hand, there is no strong evidence supporting an allosteric model, either. Therefore, in this study, we do not intend to determine which of the two models, an allostric model and the two-sensor model, is correct. Rather an important point is that we could simulate transmitter release evoked by the AP-like
stimulation using the transmitter release models, assuming that cAMP changes in the intracellular Ca²⁺ sensitivity and increased the RRP size (20%).

Although phorbol esters and forskolin seem to have a similar effect on transmitter release, perhaps one aspect that differs from the phorbol ester effect is that the intracellular Ca²⁺ sensitivity of only the fast-releasing vesicles is modulated. Priming of synaptic vesicles has two steps, molecular priming, which makes the vesicles fusion-competent, and positional priming, which positions the synaptic vesicles close to the Ca²⁺ channel cluster, most likely corresponding to the specific release sites within active zones (Neher & Sakaba, 2008). The fast-releasing vesicles are those that are molecularly and positionally primed whereas the slowly releasing vesicles are only molecularly primed. From the present study, it has to be postulated that cAMP ‘super primes’ vesicles (Schlüter et al. 2006) via the protein complex at the active zones. For example, cAMP activates EPAC2, which in turn modulates Rim2, an active zone protein (Kashima et al. 2001). For the case of Munc13, the protein interacting with Rim (Betz et al. 2001), it may have a dual role in priming and post-priming close to the fusion step (Basu et al. 2007). While the detailed molecular mechanisms need to be explored, the present study is consistent with the hypothesis that an active zone protein complex may have the dual functions of associating synaptic vesicles with the acceptor complex at the release sites where Ca channels cluster (Wadel et al., 2007) and of superpriming of synaptic vesicles that increases Ca sensitivity for release. AMP cannot
modulate slowly releasing vesicles during the superpriming step because they are outliers from the special release sites.
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REFERENCES


FIGURE LEGENDS

FIG. 1. Potentiation of EPSCs by forskolin under a train of AP-like stimuli

A, a 100-Hz train of 20 AP-like (depolarization to +40 mV for 0.4 to 1 ms, then ramped to -80 mV for 0.8 ms) stimuli was applied, followed by a step-depolarizing pulse to deplete the remaining pool of releasable vesicles (pre-pulse to 70 mV for 2 ms followed by a repolarization pulse to 0 mV for 50 ms). From top to bottom, the stimulation protocol, presynaptic Ca\(^{2+}\) currents, EPSCs, release rates, and the cumulative release are shown. The inset shows the release rates evoked by the first AP-like stimulus both under control and forskolin conditions (black: control; grey: forskolin + IBMX, applied to all figures). B, average amount of presynaptic Ca\(^{2+}\) influx, EPSC amplitude, the peak release rate, the release probability during the first AP-like stimulus, and the calculated size of the releasable pool of vesicles. Data were normalized to the control value in a given cell pair to illustrate the magnitude of the forskolin-induced potentiation (**, \(P < 0.01\); ***, \(P < 0.001\)).

FIG. 2. Intermediate presynaptic Ca\(^{2+}\) uncaging shows that forskolin increases transmitter release rates at the calyx of Held synapse

Flash photolysis was applied to elevate [Ca\(^{2+}\)], uniformly within the terminal. 60 ms after the flash photolysis, a step-depleting pulse was applied to deplete the remaining
pool of releasable vesicles. From top to bottom, presynaptic \([\text{Ca}^{2+}]_i\), \(\text{Ca}^{2+}\) currents, EPSCs, release rates, and cumulative release are shown. In this example, the presynaptic \([\text{Ca}^{2+}]_i\) was increased to 5.27 \(\mu\text{M}\) in controls and 4.82 \(\mu\text{M}\) after forskolin application.

**FIG. 3. Less potentiation of release rates by forskolin in response to presynaptic \(\text{Ca}^{2+}\) uncaging to higher \(\text{Ca}^{2+}\) levels**

*A*, similar to Figure 2, but the presynaptic \([\text{Ca}^{2+}]_i\) was increased to >10 \(\mu\text{M}\). *B*, double-logarithmic plot of the peak release rate per vesicle evoked by flash photolysis as a function of presynaptic \([\text{Ca}^{2+}]_i\), under control and forskolin-treated conditions (left). The slope of the dose-response curve is 4.4 under control conditions, while it is 3.1 after forskolin application. In the right panel, synaptic delays were plotted as a function of \([\text{Ca}^{2+}]_i\).

**FIG. 4. Forskolin increases the frequency of miniature EPSCs at low \(\text{Ca}^{2+}\)_i**

*A*, the presynaptic terminal was pre-loaded with an EGTA/\(\text{Ca}^{2+}\) mixture and the \(\text{Ca}^{2+}\) indicator dye fura-4F, which clamped the \([\text{Ca}^{2+}]_i\) to 200 nM. Presynaptic \([\text{Ca}^{2+}]_i\) (upper panel) and postsynaptic mEPSCs (lower panel) were recorded simultaneously (black: control; grey: after forskolin application). *B*, similar to *A*, but a weak \(\text{Ca}^{2+}\)-uncaging stimulus was applied at the time point indicated (arrow) to elevate \([\text{Ca}^{2+}]_i\) to a sub-micromolar level.
FIG. 5. cAMP modulates the fast-releasing vesicles but not the slowly releasing vesicles

A, the fast-releasing vesicles were depleted by a pre-depolarizing pulse (0 mV for 10–15 ms following a pre-pulse to +70 mV for 2 ms). Following the pre-depolarizing pulse, the test pulse was applied (0 mV for 50 ms following a pre-pulse to +70 mV for 2 ms) to deplete the remaining vesicles. From top, presynaptic voltage command, presynaptic Ca$^{2+}$ currents, EPSCs, the release rates estimated by the deconvolution, and the cumulative amounts of release are shown. Black and gray traces indicate those obtained during the control period and after the application of forskolin + IBMX, respectively. 20 µM calmodulin inhibitory peptide was included in the presynaptic patch pipette. B, the same as A, but instead of the test pulse, flash photolysis was applied to probe the Ca$^{2+}$ sensitivity of the slowly releasing vesicles. Following flash photolysis, a depleting pulse was applied to deplete the releasable pool of vesicles. C, the relationship between the peak release rate per vesicle of the slowly releasing vesicles and [Ca$^{2+}$], under control (black circles) and in the presence of forskolin + IBMX (gray circles) from the experiment shown in B.

FIG. 6. A simplified allosteric model of Ca$^{2+}$-driven vesicle fusion
A, double-logarithmic plot of peak release rate per vesicle as a function of $[\text{Ca}^{2+}]_i$. The data were obtained in $\text{Ca}^{2+}$ perfusion experiments (10 nM to 500 nM), weak $\text{Ca}^{2+}$-uncaging stimulus (600 nM to 1.5 $\mu$M), and normal flash photolysis with fura-2FF (2 $\mu$M to 14 $\mu$M). The black (control) and grey (forskolin) lines were predictions from the simplified allosteric model. B, the scheme of the simplified allosteric model.

**FIG. 7. Simulating the time course of transmitter release during an AP-like stimuli using a simplified allosteric model**

A, The presynaptic terminal was stimulated by the ramp protocol, which depolarized the terminal to +40 mV and then repolarized back to -80 mV. From top, voltage command, presynaptic $\text{Ca}^{2+}$ currents, EPSCs, and release rates estimated from the deconvolution method are shown. B, The panel shows the average time course of transmitter release under control condition and the condition after application of forskolin + IBMX (solid traces). Grey dotted traces show the simulated time course of transmitter release. The parameters of an simplified allosteric model were determined in Fig 6. For simulating forskolin + IBMX data, 20 % increase in the RRP size was also taken into account.
A

- $V_{pre}$
- $I_{Ca}$
- EPSC
- Release rate
- Cumul. rel.

B

- Bar graph showing:
  - Ca influx
  - EPSC amplitude
  - Peak release rate
  - Pr
  - Pool size

- Test/control ratio:
  - $n = 9$
A

Flash

$[Ca^{2+}]_i$ (µM)

$I_{Ca}$

EPSC

release rate

cumul. rel.

$1 \text{nA}$

$5 \text{nA}$

$500 \text{ms}^{-1}$

$1000 \text{vesicles}$

$20 \text{ms}$

B

Peak release rate (vesicle ms$^{-1}$)

slope=3.1

$[Ca^{2+}]_i$ (µM)

Delay (ms)

slope=4.4

control

forskolin

control

forskolin
A

![Graph showing release rate per vesicle vs. intracellular calcium concentration](image)

- Control
- Forskolin
- Control_allosteric model
- Forskolin_allosteric model

B

![Diagram of vesicle fusion process](image)

- $V \xrightarrow{5k_{on}} V_{Ca}$
- $V_{4Ca} \xrightarrow{k_{on}} V_{5Ca}$
- $l_+ \xrightarrow{k_{off}b^0} l_+f$
- $l_+ \xrightarrow{k_{off}b^4} l_+f^4$
- $l_+ \xrightarrow{k_{off}b^5} l_+f^5$

Fused