

SUPPLEMENTAL MATERIAL

Epac activation enhances neurotransmitter release at excitatory central synapses

I. Gekel and E. Neher

a) Enhancement of neurotransmitter release by various ESCAs

To exclude a contamination of our measured EPSCs by AP-related sodium and potassium current traces due to an overlap of their time-courses we determined the AMPA receptor difference current (supplemental Fig. 1A). EPSCs were recorded in the absence or presence of CNQX (10 μ M). Subtraction of the EPSC traces recorded under both conditions yielded the AMPA receptor difference current. The amplitudes of the AMPA receptor difference currents were indistinguishable from those of the EPSCs recorded in the absence of CNQX. Therefore, the size of the measured EPSCs was not affected by the preceeding AP-related sodium and potassium current traces.

The action of ESCA₁ on neurotransmitter release from cultured excitatory autaptic neurons from the *Dentate Gyrus* was mimicked by structurally-related ESCAs (supplemental Fig. 1B1,2). Using cultures of *Trypanosoma brucei*, studies of Laxman et al. (2006) showed that ESCA₁ not only is susceptible to hydrolysis by cyclic nucleotide phosphodiesterases (PDEs) but also may inhibit the hydrolysis of endogenous cAMP by its interaction with PDEs (possibly leading to indirect activation of PKA by ESCA₁). We tested whether hydrolysis of ESCA₁ also affects the drug-induced enhancement of neurotransmitter release in mammalian cells during 0.2 Hz stimulation. Therefore, we applied Sp-8'-pCPT-2'-O-Me-cAMPS (ESCA₂), a phosphorothioate derivative of cAMP which is resistant to enzymatic hydrolysis catalyzed by PDEs, to the neuronal cultures. The mean EPSC amplitude enhancement for ESCA₂ (20 ± 2 %, mean $\pm$ SEM, N = 17) was not significantly different from the

one for ESCA₁ (25 ± 2 %, mean $\pm$ SEM, N = 49) (supplemental Fig. 1B1). To further validate that ESCA₁ acted via Epac, we performed a second experiment with the structurally-related 8'-pMeOPT-2'-O-Me-cAMP (ESCA₃). ESCA₃ was shown to displace the binding of [³H]cAMP to Epac with a higher potency than ESCA₁ (7.1-fold versus 4.6-fold) (Christensen et al., 2003). Although ESCA₃ combines the highest affinity towards Epac with the highest Epac/PKA selectivity of all ESCAs, it exhibits only a 50-fold higher lipophilicity than cAMP when compared to ESCA₁ (70-fold) or to ESCA₂ (90-fold) (Biolog, Bremen, Germany). On average ESCA₃ led to an EPSC amplitude enhancement of 22 ± 5 % (N = 19) (supplemental Fig. 1B2). The corresponding ESCA₁-effect was 28 ± 3 % (N = 34). From these observations we conclude that neither hydrolysis nor membrane-permeability of ESCA₁ led to a subestimation of its effect on neurotransmitter release during 0.2 Hz stimulation and during an application time of 1.2 minutes.

b) Role of HCN channels and PKA for neurotransmitter release

Since ESCA₁ application only partially accounted for the observed size of the forskolin-mediated increase of the EPSC amplitudes, we experimentally addressed possible contributions of both HCN channels and PKA.

When murine excitatory autaptic DG-neurons were hyperpolarized by a voltage step from -70 mV to -120 mV, neither application of the HCN channel antagonist ZD7288 (200 μ M; Gasparini and DiFrancesco, 1997) nor of CsCl (10 μ M) for two minutes inhibited hyperpolarization-activated currents (supplemental Fig. 2A1). However, both drugs led to changes in evoked release, when used at the previously mentioned concentrations. ZD7288 reduced evoked release on average by 47 ± 3 % (mean $\pm$ SEM, N = 4), CsCl slightly increased evoked release on average by

11 ± 1 % (mean ± SEM, N = 3; see supplemental Fig. 2A2 for sample traces). This may be explained by the finding that both AMPA and NMDA currents of murine dentate granule cells are subject to inhibition by ZD7288 (Huang and Hsu, 2003). In contrast, application of BaCl₂ inhibited hyperpolarization-activated currents in a concentration dependent manner (10 μM: 46 ± 7 %, mean ± SEM, N = 6; 100 μM: 65 ± 3 %, mean ± SEM, N = 2, data not shown; 500 μM: 75 %, N = 1; supplemental Fig. 2A1). BaCl₂ is known to inhibit inwardly rectifying potassium channels (Robinson and Siegelbaum, 2003). In individual cases 10 μM BaCl₂ was sufficient to completely block the hyperpolarization-activated current from excitatory autaptic DG-neurons (data not shown). Moreover, 10 μM BaCl₂ enhanced evoked release (supplemental Fig. 2A2) and mEPSC frequency (data not shown). This observation is in agreement with previous publications on the effects of BaCl₂ on neurotransmitter release (Zhang et al., 1993; Tang et al., 1994). In our experiments BaCl₂ concentrations around 500 μM and above led to a loss of the giga-seal. Therefore, we had to limit our experiments with BaCl₂ to concentrations below 500 μM. Similar observations were made for CsCl. Traditionally both ions are used in the millimolar range when analysing hyperpolarization-activated currents in neuronal slice preparations (Cuttle et al., 2001; Chevaleyre and Castillo, 2002). In summary we conclude that the observed hyperpolarization-activated current does not occur due to HCN channel activation, instead it most likely originates from inwardly rectifying potassium channels. Moreover, the effects of ZD7288 or CsCl on evoked release are not related to hyperpolarization-activated currents.

Considering the effects of ZD7288 and CsCl, our results only partially relate to the observation made by Huang and Hsu (2003) in slice preparations of murine dentate granule cells. Whereas the amplitude of hyperpolarization-activated currents

was unaltered during the first two minutes of drug application in this preparation, prolonged incubation with ZD7288 (50 μ M) or CsCl (1 mM) for 25 minutes inhibited this current by 50% (Huang and Hsu, 2003). However, HCN channel isoforms are absent or barely detectable above background at the mRNA level in murine dentate granule cells (Moosmang et al., 1999; Santoro et al., 2000). Our results suggest that HCN channel function is absent from murine excitatory autaptic neurons, and therefore any contribution to the forskolin response on evoked release is unlikely.

PKA activation by the specific cAMP analog 6-Bnz-cAMP (50 μ M) increased EPSC amplitudes from excitatory DG-neurons during low stimulation frequency (0.2 Hz). On average EPSC amplitudes were potentiated by a factor of 1.21 ± 0.07 (mean $\pm$ SEM, N = 10; supplemental Fig. 2B). From this observation and considering the size of the forskolin effect on EPSC amplitudes (factor = 1.76 ± 0.13 , mean $\pm$ SEM, N = 35) we conclude that PKA- and Epac-signaling do not show a simple additive behaviour. The forskolin effect was not inhibited by application of the PKA antagonist KT5720 (1 μ M and 4 μ M, $K_i \sim 56$ nM; supplemental Fig. 2C), although KT5720 was previously shown to inhibit cellular PKA function at the concentrations used here (Nagy et al., 2004; Huang and Hsu, 2006). In contrast, KT5720 (1 μ M) enhanced a simultaneous effect of ESCA₁ on EPSC amplitudes on average by 100 % (supplemental Fig. 2C). These results unfortunately are complex and do not help to separate the roles of PKA-dependent and PKA-independent pathways. However, they are well compatible with an inhibitory role of PKA-mediated phosphorylation in the Epac-pathway. In any case, our new results speak against the ESCA₁-effect to be mediated via PKA.

c) Characteristics of the ESCA₁-mediated enhancement of mEPSC frequency

In the main text body of our manuscript we reported that ESCA₁ enhanced mEPSC frequency in the absence of action potential-induced calcium ion influx on average by a factor of 2.9 ± 0.5 (mean $\pm$ SEM) for young cell culture ages (DIV8-10) (Fig. 4). For young cell culture ages (DIV9-10) mEPSC frequency was also enhanced in the last 900 ms of EPSC traces (supplemental Fig. 2A1) recorded every five seconds (0.2 Hz), albeit to a lesser extent (1.9-fold on average). In the supplemental Fig. 2A2 we show that the degree of mEPSC frequency enhancement depends on calcium ion influx. We considered only recordings from young cell culture ages to assure that the values from different cultures belong to the same distribution.

After ESCA₁ application the effect on mEPSC frequency decayed monoexponentially with a time constant of $\tau \sim 25 \pm 1$ seconds (mean $\pm$ SD) during 0.2 Hz stimulation (supplemental Fig. 2A1), whereas the ESCA₁-effect on EPSC amplitudes decreased with a time constant of $\tau \sim 13 \pm 1$ seconds (mean $\pm$ SD) (Fig. B1). This observation complements our results presented in Fig. 6.

Finally, we tested for alterations in paired-pulse facilitation. Since the ESCA₁-induced increase in mEPSC frequency could be as well considered as being the result of a postsynaptic ‘unsilencing’ of silent synapses, analysis of paired-pulse behavior will provide another line of evidence for the ESCA₁-mediated increase in release probability. The paired-pulse experiment was performed in the presence of low external calcium concentration (0.75 mM) to lower release probability. Release probability shapes the paired-pulse ratio: low release probability is generally believed to enhance facilitation, whereas high release probability will result in overall depression (Thomson, 2000).

In the presence of ESCA₁ the paired-pulse ratio was reduced when compared to control condition (supplemental Fig. 3B). This reduction occurred due to the ESCA₁-mediated increase of the first pulse (p1), whereas the second pulse (p2) remained largely unchanged upon drug-treatment. In case of postsynaptic ‘unsilencing’ of synapses no change in facilitation is expected. Thus, the paired-pulse experiment showed that a postsynaptic ‘unsilencing’ effect is unlikely to account for the observed effect of ESCA₁ on mEPSC frequency.

d) Persistence of the ESCA₁ effect after prolonged preincubation with PDBu

Potentiation of neurotransmitter release by acute ESCA₁ application persisted after prolonged PDBu pretreatment for 16h (supplemental Fig. 3). After control preincubation with DMSO only, ESCA₁ enhanced EPSC amplitudes on average by $21 \pm 4 \%$ (mean $\pm$ SEM, N = 9). After preincubation with PDBu for at least 16 hours the potentiation by ESCA₁ was $18 \pm 4 \%$ (mean $\pm$ SEM, N = 14). In contrast, the PDBu effect on EPSC amplitudes was significantly ($p < 0.001$) reduced by $101 \pm 2 \%$ (mean $\pm$ SEM, N = 8). Mean EPSC₀ values were not different between control measurements (1.88 ± 0.36 nA, mean $\pm$ SEM, N = 15) and those after PDBu pretreatment (1.54 ± 0.36 nA, mean $\pm$ SEM, N = 8). Moreover, ESCA₁ did not lead to a PDBu effect upon subsequent PDBu application in PDBu pretreated neurons (IAI = 2 min). Therefore, Epac function itself does not rely on PKC activity which can be downregulated by prolonged pretreatment with PDBu.

Our results suggest that the fast ESCA₁ effect presumably does not rely on PKC/Munc18-1 activity (*see Discussion*). The persisting effect on ESCA₁ may depend, however, on PKC/Munc18-1 activity.

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FIGURE LEGENDS

Supplemental Fig. 1 Influence of AP-related currents, ESCA₂ (hydrolysis-resistant) and ESCA₃ (higher Epac affinity) on neurotransmitter release from excitatory autaptic DG-neurons. **A.** Sample traces for the AMPA receptor difference current. EPSCs were recorded in the absence or presence of CNQX (10 μ M). Subtraction of the EPSC traces recorded under both conditions yielded the AMPA receptor difference current. $\Delta(\text{control})_{\text{CNQX}} = \text{EPSC}_{\text{control}} - \text{EPSC}_{\text{CNQX}}$; $\Delta(\text{Forskolin})_{\text{CNQX}} = \text{EPSC}_{\text{Forskolin}} - \text{EPSC}_{\text{Forskolin+CNQX}}$ **B1.** Average EPSC amplitude potentiation by 50 μ M ESCA₁ (black bar) and 50 μ M ESCA₂ (Sp-8'-pCPT-2'-O-Me-cAMPS, hydrolysis-resistant ESCA analogue; white bar). ESCA₁: N = 49, n = 10 (EPSC₀ = 3.68 $\pm$ 0.45 nA, mean $\pm$ SEM); ESCA₂: N = 17, n = 1 (EPSC₀ = 3.68 $\pm$ 0.29 nA, mean $\pm$ SEM). **B2.** Average EPSC amplitude potentiation by 50 μ M ESCA₁ (black bar) and 50 μ M ESCA₃ (8'-pMeOPT-2'-O-Me-cAMP, ESCA analogue with an approximately 1.5-fold higher affinity towards Epac than ESCA₁; white bar). ESCA₁: N = 34, n = 8 (EPSC₀ = 2.53 $\pm$ 0.21 nA, mean $\pm$ SEM); ESCA₃: N = 19, n = 2 (EPSC₀ = 2.35 $\pm$ 0.30 nA, mean $\pm$ SEM). Similar EPSC₀ amplitude distributions were considered for ESCA₁-treated and ESCA₂/ESCA₃-treated cells. Statistical significance of the individual drug-induced EPSC potentiations was analysed by the paired *t*-test. For comparison between the ESCA₁- & the ESCA₂- or the ESCA₃-effect the KS-test was used. The numbers within the histogram bars are N-values, representing the number of cells.

Supplemental Fig. 2 Role of HCN channels and PKA for neurotransmitter release. **A1.** Sample traces of hyperpolarization-activated currents recorded in the absence (black) or presence (grey) of various inhibitors such as: 200 μ M ZD7288, 10

μM CsCl, 10 μM and 500 μM BaCl₂. Autaptic DG-neurons were hyperpolarized by a voltage step from the membrane potential of -70 mV to -120 mV. ZD7288 and CsCl are known as HCN channel blockers. BaCl₂ inhibits inwardly rectifying potassium channels. **A2.** EPSC sample traces prior (black) and after (grey) modulation by the previously mentioned inhibitors. EPSC and hyperpolarization-activated currents were both recorded from the same autaptic neuron. Inhibitor-induced changes in the EPSC amplitudes serve as positive controls for effective drug concentrations. **B.** Average EPSC amplitude potentiation by 50 μM forskolin (black bar; EPSC₀ = 3.90 ± 0.42 nA, mean ± SEM), 50 μM 6-Bnz-cAMP (grey bar; Bnz, EPSC₀ = 3.38 ± 1.01 nA, mean ± SEM) and 50 μM ESCA1 (white bar; EPSC₀ = 4.30 ± 0.34 nA, mean ± SEM). Values are shown as mean ± SEM. Statistical significance of the individual drug-induced EPSC potentiations was analysed by the paired *t*-test. Significance in-between the different drug-effects was analysed by the KS-test. **C.** Change in the forskolin and ESCA₁ response by the PKA inhibitor KT5720 (Δ Potentiation). a, b: % change in the average EPSC amplitude potentiation by 50 μM forskolin in the presence 1 μM or 4 μM KT5720, respectively (EPSC₀ = 1.91 ± 0.65 nA, mean ± SEM). c: % change in the average EPSC amplitude potentiation by 50 μM ESCA₁ in the presence of 1 μM KT5720 (EPSC₀ = 2.45 ± 0.34 nA, mean ± SEM). The numbers in brackets are N-values, representing the number of cells. Statistical significance of the individual KT5720-induced changes of the forskolin and ESCA₁ response was analysed by a paired *t*-test.

Supplemental Fig. 3 Characteristics of the ESCA₁-mediated enhancement of mEPSC frequency. A1. Dependency of the normalized mEPSC frequency (mean ± SEM) on time: The number of mEPSC events from the last 900 ms of three

subsequent traces (each of 1 s length) recorded at 0.2 Hz were counted and grouped together (to reduce the number of datapoints and to increase in parallel the number of detected mEPSC events) (exception: the first point during ESCA₁ application was calculated only from the first trace). From the number of mEPSC events the mEPSC frequency was calculated for three stimulation conditions (prior, during and after ESCA₁ stimulation). The mEPSC frequency values were normalized to the average mEPSC frequency determined prior to ESCA₁ stimulation. To assure that the values from different cultures belong to the same distribution we considered only recordings performed on DIV 9 and 10 (average DIV = 9.4). ● control; ▷ ESCA₁(50 μM); N = 25, n = 6 (EPSC₀ = 3.5 ± 0.4 nA, mean ± SEM). For the cells analysed the average EPSC amplitude potentiation by ESCA₁ was 28 ± 3 % (mean ± SEM) **A2**. Timecourse of the mEPSC frequency in the presence (0.2 Hz) or absence (TTX) of action potential-stimulated calcium ion influx from the same cell. During treatment with TTX no depolarization pulses were elicited. The mEPSC frequency values were normalized to those obtained for tetrodotoxin (TTX) treatment only. For the TTX treatment a single mEPSC frequency datapoint contains the mEPSC events detected within a time window of 15 seconds. mEPSC frequency from 0.2 Hz recordings was determined as described in part A. We considered only recordings performed on DIV 9 and 10 (average DIV = 9.6). ● control; □ TTX(300 nM); ▷ ESCA₁(50μM); N = 18, n = 2 (EPSC₀ = 2.4 ± 0.2 nA, mean ± SEM). **B**. Sample trace for autaptic paired-pulse facilitation in an external solution containing 0.75 mM Ca²⁺ and 2.25 mM Mg²⁺. Paired pulses were recorded from DG-neurons at an interstimulus interval (ISI) of 20 ms either under control condition (black traces) or during ESCA₁ stimulation (grey traces). Prior to paired-pulse recording in the presence of ESCA₁ the autapse was prestimulated for 1.2 minutes with ESCA₁. Only neurons which did not

show a rundown of their EPSC amplitudes in-between paired-pulse recordings during control and drug condition were analysed.

Supplemental Fig. 4 ESCA₁ effect on neurotransmission after prolonged neuronal pretreatment with PDBu. Average (ave.) potentiation of the EPSC amplitudes by ESCA₁ in the presence (+ 16 h PDBu) or absence (- 16 h PDBu) of preincubation with PDBu for 16 hours (mean $\pm$ SEM). Significance was tested by a KS-test. The recordings for both conditions were not obtained from identical cells. Therefore, we generated two different control values (black bars) for the mean ESCA₁ effect. *m*: ESCA₁ potentiation values measured in the absence of PDBu preincubation (N = 32, n = 6). *d*: ESCA₁ potentiation values after preincubation with DMSO for 16 h (volume of application corresponded to the one used for pretreatment with PDBu; N = 9, n = 2). For the pretreatment condition (+ 16 h PDBu; white bar): N = 14, n = 2. Both, the control and the PDBu preincubation condition show similar mean EPSC₀ values (2.6 ± 0.3 nA versus 1.9 ± 0.4 nA, mean $\pm$ SEM). The numbers within the histogram bars are N-values, representing the number of cells.