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Rapid changes in hippocampal CA1 pyramidal cell function via pre- as well as postsynaptic membrane mineralocorticoid receptors

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Abstract

Corticosterone (100 nM) rapidly increases the frequency of miniature excitatory postsynaptic currents in mouse CA1 pyramidal neurons via membrane-located mineralocorticoid receptors (MRs). We now show that a presynaptic ERK1/2 signalling pathway mediates the nongenomic effect, as it was blocked by the MEK inhibitors U0126 (10 μM) and PD098059 (40 μM) and occluded in *H-Ras*^{G12V}-mutant mice with constitutive activation of the ERK1/2 presynaptic pathway. Notably, the increase in mEPSC frequency was not mediated by retrograde signalling through endocannabinoids or nitric oxide, supporting presynaptic localization of the signalling pathway. Unexpectedly, corticosterone was also found to have a direct postsynaptic effect, rapidly decreasing the peak amplitude of I_A currents. This effect takes place via postsynaptic membrane MRs coupled to a G protein-mediated pathway, as the effect of corticosterone on I_A was effectively blocked by 0.5 mM GDP-β-S administered via the recording pipette into the postsynaptic cell. Taken together, these results indicate that membrane MRs mediate rapid, nongenomic effects via pre- as well as postsynaptic pathways. Through these dual pathways, high corticosterone concentrations such as occur after stress could contribute to enhanced CA1 pyramidal excitability.

Introduction

The adrenal stress hormone corticosterone, after binding to mineralocorticoid (MR) and glucocorticoid (GR) receptors in the hippocampus, modifies transcription of responsive genes via DNA-binding of receptor homodimers or interactions with transcription factors (Aranda & Pascual, 2001). These actions slowly but persistently alter hippocampal cell properties including voltage-dependent calcium currents (Karst *et al*., 2000). Recently it has become clear that corticosterone not only induces (long-lasting) cellular changes through genomic actions but also affects cellular physiology faster, via nongenomic pathways (Di *et al*., 2003, 2005; Karst *et al*., 2005). In the hypothalamus these rapid effects are mediated via receptors resembling GRs, stimulating synthesis and release of endocannabinoids which then act as retrograde messengers (Di *et al*., 2003; Malcher-Lopes *et al*., 2006). In the hippocampus, though, MRs rather than GRs have been shown to mediate rapid effects (Karst *et al*., 2005).

Thus, in CA1 pyramidal cells corticosterone enhances the frequency of miniature excitatory postsynaptic currents (mEPSCs) via membrane-located MRs (Karst *et al*., 2005). No change in amplitude or in kinetic properties was observed. As the first relative to the second response in a double-pulse stimulation protocol was enhanced by corticosterone, it was inferred that corticosterone increases the release probability of glutamate-containing vesicles (Karst *et al*., 2005); this agrees with an earlier microdialysis study (Venero & Borrell, 1999). This enhancement of glutamate transmission could have implications for the role of corticosterone and stress in hippocampal synaptic plasticity and related processes such as learning (Kim & Diamond, 2002; Shors, 2006; Joëls *et al*., 2008), in line with the notion that scaling of mEPSC frequency is thought to be involved in activity-dependent refinement of neuronal connectivity (Desai *et al*., 2002). At present, though, the signalling pathway underlying the rapid MR effects in brain is entirely unknown.

One possible mediator involved in the nongenomic MR pathway is the extracellular signal-regulated kinase (ERK)1/2, which modulates synaptic plasticity (Sweatt, 2004) and is implicated in learning (Zhang *et al*., 2004; Kushner *et al*., 2005). Mutant mice with a constitutively activated presynaptic ERK1/2 pathway display enhanced mEPSC frequency in CA1 pyramidal neurons (Kushner *et al*., 2005), similar to the rapid effects of corticosterone, suggesting that MRs could be upstream activators of ERK1/2. The ERK1/2 pathway is modulated by various signalling molecules, such as G proteins and endocannabinoids (Derkinderen *et al*., 2003). We therefore hypothesized that corticosterone, via a G protein-coupled receptor, regulates ERK1/2 activity and thus modulates glutamate transmission. Our data indeed support this. In contrast to rapid effects of corticosterone in the hypothalamus, the MR signalling cascade leading to enhanced mEPSC frequency appears to be entirely presynaptically located. However, the postsynaptic ERK1/2 pathway is also affected by corticosterone, leading to attenuation of a K^+-conductance. Taken together, these findings indicate that membrane MRs are most probably located on both the pre- and the postsynaptic sides of CA1 pyramidal cell synapses. Potentially, through these dual MR-mediated pathways, rises in corticosterone

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level such as occur after stress could rapidly enhance the excitability of CA1 neurons.

Materials and methods

All experiments, unless stated otherwise, were performed in male C57/Bl6 mice, 6–8 weeks of age (group-housed), with permission of the Universiteit van Amsterdam Animal Committtee (DEC). Food and water were given *ad libitum*. One mouse per day was decapitated under rest, between 09.00 and 09.30 h, when circulating plasma corticosterone levels are low (Karst *et al*., 2000). A limited number of experiments was performed in male *H-Ras*^{G12V} mice (also on a C57/Bl6 background; Kushner *et al*., 2005).

On the day of the experiment, the animal was decapitated and the brain was quickly removed from the skull and transferred to ice-cold, continuously gassed (95% O_2, 5% CO_2) artificial cerebral spinal fluid (aCSF) containing (in mM): NaCl, 120; KCl, 3.5; $MgSO_4$, 1.3; NaH_2PO_4, 1.25; $CaCl_2$, 2.5; $NaHCO_3$, 25; and glucose, 10. Hippocampal slices (350 μM thick) were made with a vibratome (model VT1000S; Leica, Germany) and stored at room temperature (for at least 1 h). One slice at a time was fully submerged in a custom-made recording chamber (volume ∼2 mL) mounted on an upright microscope fitted with differential interference contrast (Zeiss Axioskop, Germany) and continuously perfused with aCSF (32°C, 2–3 mL/min). The aCSF temperature was controlled and monitored continuously with an automated chamber heater (Warner, USA). Bicuculline methchloride (20 μM; Tocris, UK) and tetrodotoxin (0.5 μM; Latoxan, France) were added to block $GABA_A$ receptor-mediated signals and action potentials, respectively.

A cleaning pipette removed debris from the surface of the CA1 cell layer and CA1 pyramidal neurons were visually identified. Whole-cell voltage-clamp recordings were made with an Axopatch 200B amplifier (Axon Instruments, USA) using borosilicate glass electrodes (impedance 4–6 MΩ, 1.5 mm outer diameter; Hilgenberg, Germany) pulled with a micropipette puller (Brown/Flaming P-87; Sutter Instruments, USA). Series resistance compensation was > 70% in all recordings.

For mEPSC recordings the intracellular pipette solution contained (in mM): Cs methane sulphonate, 120; CsCl, 17.5; HEPES, 10; BAPTA, 5; MgATP, 2; Na guanosine triphosphate (GTP), 0.1; and QX-314, 10; osmolarity 295 mOsm, pH 7.4 adjusted with CsOH. BAPTA was obtained from Molecular Probes (the Netherlands) and the sodium channel blocker QX-314 from Alomone (Israel); all other chemicals were purchased from Sigma, the Netherlands. All mEPSCs were recorded under a holding potential of −70 mV. Only recordings with uncompensated series resistance of < 2.5× the pipette resistance were accepted for analysis. In a restricted number of cells we examined the effect of corticosterone on miniature inhibitory postsynaptic current (mIPSC) properties. In these experiments the intracellular pipette solution contained (in mM): CsCl, 141; HEPES, 10; EGTA, 10; MgATP, 2; NaGTP, 0.1; and QX-314, 10; osmolarity 280 mOsm, pH 7.2 adjusted with CsOH. Instead of bicuculline, 10 μM CNQX and 10 μM D-AP5 were added to the perfusion solution to block AMPA- and NMDA-receptor mediated currents, respectively (all drugs from Sigma, the Netherlands).

For I_A current recordings the intracellular pipette solution contained (in mM): K-gluconate, 140; HEPES, 10; EGTA, 5; $MgCl_2$, 2; $CaCl_2$, 0.1; MgATP, 2; and NaGTP, 0.1; pH 7.3 adjusted with KOH. All chemicals were obtained from Sigma, the Netherlands. All I_A currents were recorded from a holding potential of −70 mV; series resistance compensation was applied and we corrected for the liquid junction potential (∼ 5 mV). The voltage dependence of the I_A current was determined using two voltage protocols. Protocol 1 activated both the I_A and the sustained potassium current and consisted of a hyperpolarizing step to −130 mV (100 ms), followed by depolarizing steps ranging from −60 mV to +40 mV (200 ms each). Protocol 2 activated only the sustained current and consisted of a hyperpolarizing step to −130 mV (100 ms) followed by a 75-ms depolarizing step to −65 mV, followed by voltage steps similar to protocol 1. The intermediate step to −65 mV served to inactivate the transient I_A current (e.g. Numann *et al*., 1987). Subtracting protocol 2 from protocol 1 yielded the isolated I_A current. Activation curves were fitted with a Boltzmann sigmoidal curve using the equation: $I/I\text{max} = 1/(1 + \exp\ (V_t - V_{½})/\text{Slope}))$, where V_t is the tested voltage. $V_{½}$ (the voltage at which half of the current is activated) was determined from the Boltzmann fit. A change of < 10% in series resistance during recording of I_A was considered acceptable. Any cell showing a larger change was discarded from analysis. This excludes the possibility that a decrease in I_A amplitude was due to concomitant increase in effective series resistance.

Corticosterone was obtained from Sigma (the Netherlands); a fresh stock solution was made daily in ethanol. N(G)-nitro-L-arginine methyl ester (L-NAME) (Tocris, UK) and cadmium chloride (Sigma) were directly added to the aCSF. Stock solutions of 1, 2-bis-(o-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM) (Sigma) and SR141716A (gift from Solvay Pharmaceuticals, the Netherlands) were daily made in DMSO. Stock solutions of U0126 (Sigma), U0124, PB98059 (both Calbiochem) and phorbol 12-myristate 13-acetate (TPA; Tocris, UK) were made in DMSO and stored in the freezer until use. Spironolactone (Tocris) stock solution was made daily in chloroform. Final concentrations of vehicle never exceeded 0.01%. All control experiments were carried out with the vehicle concentrations in aCSF as used for the highest dose of the (ant)agonists. As reported earlier, we did not see any effect of ethanol treatment on mEPSC frequency (see Karst *et al*., 2005); values observed in DMSO (mean ± SEM, 0.53 ± 0.05 Hz, $n = 19$; not used in the previous study) were very similar to, e.g. ethanol conditions (0.56 ± 0.05 Hz, $n = 21$).

Data acquisition was carried out with PClamp (version 9.2; Axon Instruments) and analysis was performed using Clampfit (version 9.2). Minimal cut-off for mEPSC analysis was 6 pA. All values shown in the Results section are the average ± SEM of the data obtained during the final 3 min of drug application or vehicle treatment. Statistical analysis between control and a single treatment on a given group of cells was carried out using a Student's *t*-test (paired when applicable). If more than two conditions were investigated on a given set of cells, we applied a general linear model for repeated measures (within-subjects contrast). Comparison of several conditions between sets of cells was performed with ANOVA, followed by *post hoc* multiple comparisons of the mean (Tukey). In all cases, $P < 0.05$ indicated significance.

Results

Involvement of the ERK pathway

Bath application of 100 nM corticosterone (5 min) rapidly induced a 60% increase in mEPSC frequency relative to baseline in the same cells ($n = 5$; Table 1), without affecting mEPSC amplitude or kinetic properties such as time constant of decay (Table 1). Intracellular corticosterone application (100 nM) did not affect mEPSC frequency compared to the cells recorded without corticosterone in the pipette (Table 1). No difference in mEPSC frequency was observed between an early (5–10 min) and later period (10–15 min) after establishment of the whole-cell configuration ($P < 0.05$; data not shown). While the latter compared to the former is more likely to present the condition in

TABLE 1. Rapid effects of extracellularly or intracellularly administered corticosterone on hippocampal CA1 cell mEPSC and mIPSC parameters

	Frequency (Hz)	Amplitude (pA)	Decay τ (s)
mEPSC ($n = 5$)			
Vehicle control	0.65 ± 0.06	−26.6 ± 1.4	14.5 ± 1.1
Extracellular corticosterone	1.04 ± 0.10**	−26.0 ± 1.3	14.6 ± 1.3
mEPSC ($n = 5$)			
Intracellular corticosterone	0.54 ± 0.07	−28.6 ± 1.5	17.2 ± 1.5
mEPSC ($n = 5$)			
Vehicle control	0.75 ± 0.05	−26.1 ± 2.3	16.2 ± 1.0
Extracellular spironolocatone	0.76 ± 0.04	−26.1 ± 1.9	16.1 ± 1.2
Extracellular corticosterone + spironolocatone	0.77 ± 0.03	−26.9 ± 4.7	16.6 ± 2.2
mIPSC ($n = 4$)			
Vehicle control	2.45 ± 0.39	−41.4 ± 2.4	14.3 ± 0.9
Extracellular corticosterone	2.63 ± 0.28	−41.6 ± 4.8	15.3 ± 1.4

Data are presented as the mean values ± SEM, and are based on mEPSCs during the final 3 min of drug application or vehicle treatment. Corticosterone enhanced mEPSC frequency significantly (**$P < 0.01$, paired Student's *t*-test compared to vehicle control). When corticosterone was presented intracellularly via the pipette solution (Intrac. CORT, n = 5), mEPSC frequency was not elevated compared to control (unpaired *t*-test, $P = 0.20$). Recording with corticosterone in the pipette commenced at > 10 min after whole-cell configuration was established. In a third set of cells ($n = 5$), extracellularly administered corticosterone was found to be ineffective in the presence of spironolocatone; spironolactone itself did not affect mEPSC properties compared to baseline control in the same cells (ANOVA, $F = 0.027$). No significant effects were observed with respect to mEPSC amplitude or τ of decay for any of the groups. Corticosterone did not affect the properties of the mIPSCs.

which corticosterone had diffused into the cell, this can be seen as additional evidence that intracellular corticosterone did not influence mEPSC frequency. Perfusion with the selective MR antagonist spironolactone (100 nM) by itself did not change mEPSC frequency compared to the baseline recording, but prevented the enhancement of mEPSC frequency induced by extracellularly applied corticosterone (Table 1). The effect of corticosterone on mEPSC displayed selectivity, as mIPSCs recorded in the same preparation were unaffected by corticosterone (Table 1). Collectively, these data support and extend previous observations (Karst *et al*., 2005) that corticosterone specifically enhances mEPSC frequency of CA1 pyramidal neurons via a recognition site that can only be approached via the extracellular compartment, involving the MR.

To test whether rapid corticosteroid effects involve the ERK1/2 pathway we inhibited mitogen-activated protein kinase/ERK kinase (MEK), an upstream activator of ERK1/2. The potent and highly selective MEK inhibitor U0126 binds to MEK in both the active and inactive state, thus completely blocking ERK1/2 phosphorylation (Favata *et al*., 1998; Richards *et al*., 2001). We observed that U0126 significantly decreases mEPSC frequency compared to baseline control (Fig. 1), signifying that endogenously active MEK contributes to the maintenance of mEPSC frequency. Neither the mEPSC amplitude nor the tau of decay were affected (Table 2). In the presence of U0126, 100 nM corticosterone failed to enhance mEPSC frequency (Fig. 1). The inactive analogue U0124 (Favata *et al*., 1998; 10 μM) did not affect basal mEPSC frequency or the effectiveness of corticosterone in enhancing the frequency (Fig. 1). We also tested the MEK inhibitor PD098059 (40 μM), which only binds the inactive state of MEK and substantially but not completely prevents ERK1/2 phosphorylation (Favata *et al*., 1998; Richards *et al*., 2001). PD098059 did not affect basal mEPSC frequency (consistent with the fact that this substance only binds to inactive MEK) but prevented the corticosterone-induced enhancement of mEPSC frequency: as is shown in Fig. 1, mEPSC frequency was not significantly enhanced by corticosterone in the presence of PD098059 ($P > 0.1$). The traces in Fig. 1 furthermore show that corticosterone-induced changes in mEPSC frequency were reversible and that even with extensive drug application paradigms no gradual (spontaneous) changes in mEPSC frequency were discerned.

We next investigated whether strong activation of the ERK1/2 pathway leads to occlusion of rapid corticosteroid effects. To this end ERK phosphorylation was stimulated indirectly (see, e.g., Lange-Carter & Johnson, 1994; Yuan *et al*., 2002) with TPA (40–1000 nM). TPA enhanced mEPSC frequency in a concentration-dependent manner, by 128% for the highest dose (compared to the baseline values; $n = 5$, $P < 0.01$; Fig. 2). Subsequent application of corticosterone in the presence of TPA (1 μM) did not result in a further enhancement of the mEPSC frequency (Fig. 2). In combination with the experiments using MEK inhibitors the findings with TPA support a critical role for the MEK–ERK1/2 pathway in the enhancement of mEPSC frequency by corticosterone; the experiments with TPA by themselves are not conclusive, though, with respect to potential upstream activators of ERK1/2.

Corticosterone effects on the postsynaptic ERK1/2 pathway

After establishing the involvement of the ERK1/2 pathway, we investigated whether the ERK1/2 pathway was activated on the presynaptic or postsynaptic side. As a recent study showed that glucocorticoids in the hypothalamus can nongenomically affect the release probability of glutamate-containing vesicles by stimulating postsynaptic endocannabinoid synthesis and release (Malcher-Lopes *et al*., 2006), serving as a retrograde messenger, we first considered the possibility that in the hippocampus too the primary action of corticosterone takes place postynaptically. This predicts that MR signalling pathways involve postsynaptic G proteins activating the ERK1/2 pathway, leading to activation of multiple ERK1/2 substrates, including (i) retrograde messengers that enhance release probability of glutamate-containing vesicles, as well as (ii) the K-conductance I_A, a postsynaptic ERK1/2 substrate (Adams *et al*., 2000; Yuan *et al*., 2002; Schrader *et al*., 2006).

We first tested whether inhibiting postsynaptic G protein activity affected the enhancement of mEPSC frequency by corticosterone. Guanosine diphosphate (GDP)-β-S (0.5 mM), a nonhydrolysable GDP analogue that irreversibly inactivates G proteins, was added to the intracellular solution (replacing NaGTP) and allowed to diffuse into the patched cell for 15 min. Subsequently, corticosterone was applied for 5 min. Under those circumstances, corticosterone still enhanced the mEPSC frequency by 59 ± 7% (1.35 ± 0.22 Hz, $n = 7$) compared to baseline (0.85 ± 0.10 Hz, $P < 0.01$), excluding involvement of postsynaptic G proteins in mEPSC frequency enhancement.

However, it is still possible that there is activation of postsynaptic pathways which do not involve G proteins but which, through retrograde messengers, affect presynaptic release probability. Hence, we also tested the putative involvement of endocannabinoids as retrograde messengers in the effect of corticosterone on mEPSC frequency, as (i) presynaptic CB1 receptors regulate glutamate release (Kawamura *et al*., 2006) and (ii) endocannabinoid signalling can modulate ERK1/2 pathway activity (Zhang *et al*., 2004). The CB1 receptor antagonist SR141716A (15 min, 1 μM) alone enhanced mEPSC frequency by 59 ± 10% compared to (vehicle) baseline ($n = 4$, $P < 0.05$; Fig. 3A), suggesting residual endogenous CB1 receptor activity in our slice preparation. However, SR141716A did not interfere

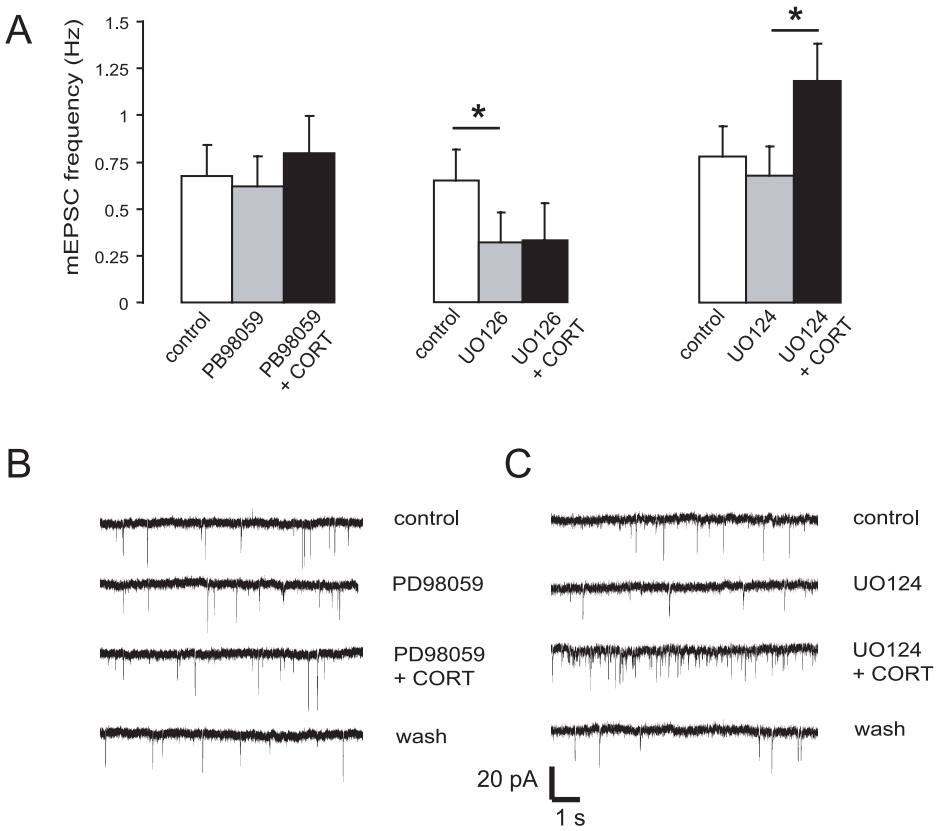

FIG. 1. Inhibition of the ERK1/2 pathway interfered with the corticosterone-induced enhancement of mEPSC frequency. (A) mEPSCs were observed during 5 min under vehicle-only conditions (DMSO; control, white bars), followed by at least 10 min application of the MEK inhibitors PB98059 (40 μM), U0126 (10 μM) or the inactive analogue U0124 (10 μM; all depicted by grey bars). Application of PB98059, U0126 or U0124 was continued for another 5–10 min but now in the presence of 100 nM corticosterone (MEK inhibitor + CORT; dark bar). PB98059 (left), which binds to the inactive form of MEK, did not affect mEPSC frequency. In the presence of this MEK inhibitor corticosterone did not result in a significant change in the mEPSC frequency. U0126 (middle), which binds to both the inactive and active form of MEK, by itself significantly decreased mEPSC frequency compared to the level seen with the DMSO vehicle. In the presence of this blocker too, corticosterone was completely ineffective in changing the mEPSC frequency. By contrast, U0124 (the inactive analogue of U0126; right) by itself did not affect mEPSC frequency and was unable to prevent a significant increase in mEPSC frequency by corticosterone. Bars represent mean + SEM; $*P < 0.01$ (for statistical analysis see Materials and methods). Data are based on mEPSCs during the final 3 min of drug application or vehicle treatment. For all recordings, mEPSC amplitudes and kinetics remained unchanged (see Table 2). (B) Original traces (from top to bottom) obtained under control conditions, in the presence of the MEK inhibitor PB98059, after addition of corticosterone and 10 min after washout of corticosterone. In the presence of the MEK inhibitor, corticosterone did not evoke any change in mEPSC frequency. These typical examples show that recordings were very stable and that no spontaneous increase or decrease in the mEPSC frequency was observed. (C) Original traces (from top to bottom) obtained under control conditions, in the presence of U0124 (the inactive analogue of the MEK inhibitor U0126), after addition of corticosterone and 10 min after washout of corticosterone. A clear increase in the mEPSC frequency can be discerned during corticosterone application. Upon washout of the hormone, the mEPSC frequency returned to the pretreatment level.

with the effectiveness of corticosterone in enhancing the mEPSC frequency further (by 60% compared to SR141716A alone; $P < 0.01$), indicating that endocannabinoids acting through CB1 receptors were not critically involved in the MR effect on mEPSC frequency.

We also investigated whether NO, another retrograde signalling molecule known to affect neurotransmitter release (Keynes & Garthwaite, 2004), is involved in the corticosterone effect on mEPSC frequency, using the NO synthase inhibitor L-NAME (50 μM). L-NAME alone (10–15 min) did not significantly change mEPSC frequency compared to baseline control (Fig. 3B), but application of corticosterone in the continued presence of L-NAME enhanced mEPSC frequency by 78 ± 20% compared to L-NAME alone ($n = 4$, $P < 0.01$), arguing against involvement of the retrogradely activated NO signalling pathway. Collectively these experiments suggest that the rapid MR-induced ERK1/2 activation leading to increased glutamate release probability did not occur via postsynaptic signalling pathways involving retrograde messengers.

Does this mean that rapid MR effects do not alter the postsynaptic ERK1/2 signalling pathway at all? To test this, we examined whether corticosterone application affects the K-conductance I_A, a well-documented substrate for the postsynaptic ERK1/2 pathway (Adams *et al.*, 2000; Yuan *et al.*, 2002; Schrader *et al.*, 2006). We used two activation protocols (Fig. 4A and B) which after subtraction yield the I_A in isolation. Brief (5 min) application of 100 nM corticosterone decreased the peak amplitude of I_A currents compared to (vehicle) baseline before corticosterone application, while activation of sustained potassium currents was unaffected (Fig. 4A and B). Further analysis showed a corticosterone-induced rightwards shift of the activation curve (Fig. 4C), as demonstrated by the change in $V_{½}$ from −23 ± 2 to −11 ± 3 mV ($n = 5$, $P < 0.01$; Fig. 4D). This resembles the effect of ERK1/2 alone (Adams *et al.*, 2000; Yuan *et al.*, 2002; Schrader *et al.*, 2006). Intracellular application of corticosterone in a separate group of cells ($n = 6$) did not affect I_A compared to control (Fig. 4D), suggesting involvement of a membrane-located receptor with an extracellular recognition site for corticosterone. In the next series the MR antagonist spironolactone was applied; this compound did not affect the $V_{½}$ compared to baseline conditions (data not shown). However, spironolactone fully blocked the corticosterone-

TABLE 2. No MEK inhibitors or activators, blockers of retrograde signalling or compounds interfering with free calcium significantly affected mEPSC amplitude or τ of decay (subsequent application of corticosterone was also ineffective)

	mEPSC	
	Amplitude (pA)	τ of decay (ms)
PB98059 ($n = 5$)		
Vehicle-only baseline	−21.4 ± 1.7	12.7 ± 0.7
PB98059	−23.1 ± 2.5	12.8 ± 0.7
PB98059 + CORT	−23.6 ± 2.5	12.8 ± 0.8
U0126 ($n = 5$)		
Vehicle-only baseline	−23.1 ± 2.7	12.4 ± 1.2
U0126	−21.2 ± 3.0	13.4 ± 1.0
U0126 + CORT	−21.9 ± 3.0	13.5 ± 1.0
U0124 ($n = 5$)		
Vehicle-only baseline	−22.5 ± 2.8	14.0 ± 0.9
U0124	−24.9 ± 2.7	14.1 ± 0.8
U0124 + CORT	−24.3 ± 2.4	13.7 ± 0.7
TPA ($n = 5$)		
Vehicle-only baseline	−22.0 ± 1.9	13.0 ± 1.3
TPA, 40 nM	−21.8 ± 2.0	12.8 ± 1.0
TPA, 200 nM	−20.6 ± 2.2	13.1 ± 1.0
TPA, 1 μM	−23.1 ± 1.8	12.3 ± 1.2
TPA, 1 μM + CORT	−24.7 ± 3.3	11.8 ± 1.7
SR141716A ($n = 4$)		
Vehicle-only baseline	−26.9 ± 2.9	15.7 ± 1.4
SR141716A	−31.2 ± 3.0	16.0 ± 1.8
SR141716A + CORT	−28.2 ± 3.0	16.7 ± 1.3
L-NAME ($n = 4$)		
Vehicle-only baseline	−24.2 ± 3.8	13.8 ± 1.2
L-NAME	−27.1 ± 4.1	13.7 ± 0.9
L-NAME + CORT	−25.1 ± 3.1	13.1 ± 1.2
BAPTA ($n = 5$)		
Vehicle-only baseline	−22.3 ± 1.6	14.4 ± 0.5
BAPTA	−22.1 ± 1.6	14.3 ± 0.6
BAPTA + CORT	−23.1 ± 1.9	15.0 ± 0.5
Cd ($n = 5$)		
Vehicle-only baseline	−26.7 ± 4.1	15.5 ± 2.7
Cd	−24.2 ± 3.8	16.2 ± 1.9
Cd + CORT	−26.3 ± 3.1	17.7 ± 1.3

All data are the mean ± SEM of mEPSCs recorded during the final 3 min of drug application or vehicle treatment; *n*, number of cells. Data analysis was performed with a general linear model for repeated measures. For details on drug application and effects on mEPSC frequency see main text.

induced decrease in I_A amplitude ($n = 5$, $P < 0.05$; Fig. 4C and D). As established in another set of cells ($n = 4$), intracellular application of 0.5 mM GDP-β-S did not affect the I_A *per se* but did prevent the corticosterone-induced change in $V_{½}$ ($P < 0.05$; Fig. 4D), indicating the involvement of a G protein-dependent pathway coupled to a membrane-located MR. All in all, these data strongly suggest that corticosterone via MRs and G-proteins activates the postsynaptic ERK1/2 pathway in hippocampal cells, thus decreasing I_A amplitude. This pathway, however, is not involved in the MR-dependent increase in mEPSC frequency.

Presynaptic effects of corticosterone

As, unexpectedly, the postsynaptic ERK1/2 pathway was not involved in the effect of corticosterone on mEPSC frequency, we examined the possible involvement of the presynaptic ERK1/2

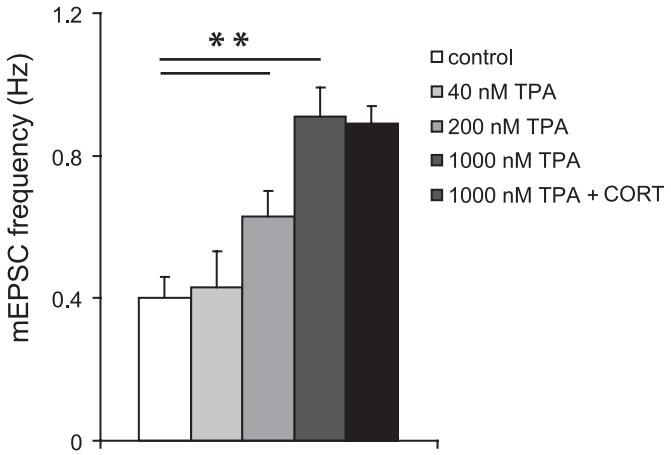

FIG. 2. Activation of the ERK pathway occluded corticosterone-induced enhancement of mEPSC frequency.The nonselective ERK activator TPA enhanced mEPSC frequency in a concentration-dependent manner. Properties of mEPSCs were first recorded under control (vehicle) conditions ($n = 5$). Subsequently, 40 nM TPA was perfused for 5–10 min, followed by 200 and then 1000 nM concentrations of TPA. The two higher concentrations resulted in an enhanced mEPSC frequency compared to baseline. As a final step, 1000 nM TPA + CORT was administered. This did not yield a different mEPSC frequency compared to 1000 nM TPA alone. Error bars represent SEM; $^{**}P < 0.01$ (for statistical analysis see Materials and methods). For all recordings, mEPSC amplitudes and kinetics remained unchanged (see Table 2).

pathway, using transgenic *H-Ras*^{G12V} mice. These mice express a constitutively active form of H-Ras (a small GTPase upstream of MEK), resulting in (i) strong activation of the presynaptic (but not postsynaptic) ERK1/2 signalling pathway, (ii) increased synapsin-I phosphorylation by ERK1/2 and (iii) increased mEPSC frequency (Kushner *et al*., 2005). If corticosterone enhances mEPSC frequency through activation of the presynaptic ERK1/2 pathway it would be expected that corticosterone would fail to do so in hippocampal slices from the transgenic mice, due to occlusion. Compared to the wild-type controls, the baseline mEPSC frequency in *H-Ras*^{G12V} CA1 pyramidal neurons was higher (Fig. 5A; Kushner *et al*., 2005). However, in the *H-Ras*^{G12V} mice, application of corticosterone failed to (further) enhance mEPSC frequency (Fig. 5A), supporting the idea that corticosterone, via a membrane-located MR, acts upstream of presynaptic ERK1/2.

To verify that in the transgenic mice mEPSC frequency could still be enhanced through pathways other than ERK1/2, a hypertonic solution of 100 mM sucrose (in regular aCSF) was applied to CA1 pyramidal neurons from *H-Ras*^{G12V} mice. Hypertonic sucrose solution stimulates the release of vesicles (independent of ERK1/2 activity), thereby greatly enhancing mEPSC frequency (Rosenmund & Stevens, 1996; Henze *et al*., 2002). Application of the sucrose solution subsequent to corticosterone in a limited number of cells ($n = 3$) enhanced mEPSC frequency in both the wild-type and transgenic mice (typical examples in Fig. 5), showing that it was still possible to enhance mEPSC frequency in *H-Ras*^{G12V} CA1 pyramidal neurons through non-ERK1/2-mediated pathways. This supports the idea that the lack of corticosteroid actions in *H-Ras*^{G12V}-mutant mice was indeed due to occlusion and not to a ceiling effect.

Although the above-described data indicate that corticosterone acts upstream of ERK1/2, corticosterone could still affect release probability through alternative presynaptic pathways, e.g. presynaptic calcium channels, similar to the gene-mediated corticosteroid actions (Karst *et al*., 2000). Therefore, we investigated whether the corticosterone-induced enhancement of mEPSC frequency was influenced by

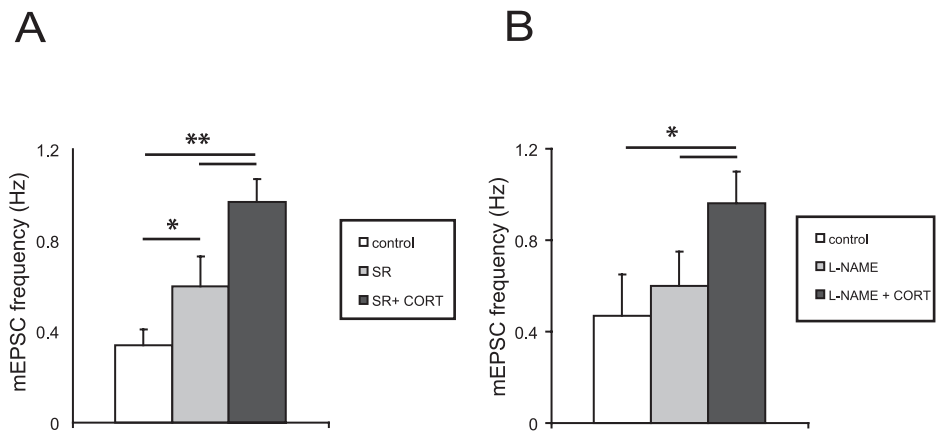

FIG. 3. Retrograde signalling via CB1 receptors or nitric oxide is not involved in the effect of corticosterone on mEPSC frequency. Properties of mEPSCs were recorded for 5 min under basal conditions (control, vehicle), followed by 10–15 min application of either the CB1 receptor antagonist SR141716A (SR; 1 μM) or the nitric oxide synthase inhibitor L-NAME (50 μM). Application of either antagonist was continued for another 5 min but now in the presence of corticosterone (CORT, 100 nM). (A) SR141716A enhanced mEPSC frequency compared to baseline (mean of $n = 4$). Continued application of SR141716A did not prevent corticosterone from further enhancing mEPSC frequency. (B) Application of L-NAME did not change mEPSC frequency, and failed to prevent corticosterone from enhancing mEPSC frequency compared to baseline ($n = 4$). Error bars represent SEM; $*P < 0.05$, $**P < 0.01$ (for description of statistical analysis see Materials and methods). For all recordings, mEPSC amplitudes and kinetics remained unchanged (see Table 2).

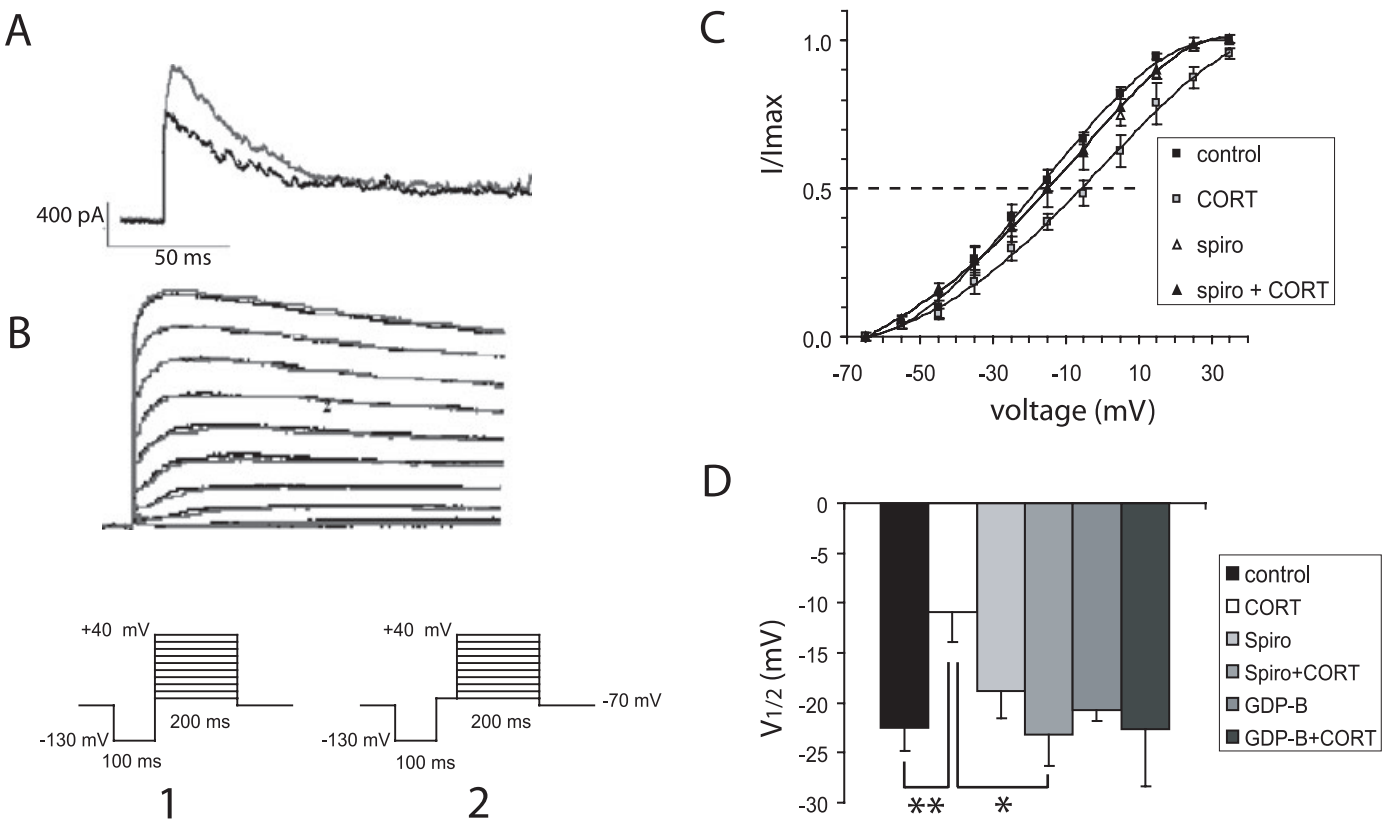

FIG. 4. Corticosterone modulated I_A currents via a postsynaptic MR and G protein. (A) Example trace of I_A currents activated using a voltage step to +40 mV. Traces were obtained by subtracting the current activated using protocol 2 from the total current activated by protocol 1. Corticosterone (100 nM, 5 min, black trace) reduced the peak amplitude of the I_A current compared to control conditions (in grey). (B) Example of the activated sustained currents using protocol 2. Corticosterone (black trace) did not change the sustained current compared to control conditions (in grey). (C) The normalized activation curves for I_A constructed from the current amplitudes elicited with voltage steps (ranging from −60 to +40 mV) and fitted with a Boltzmann equation. Corticosterone (CORT; 100 nM, 5 min, $n = 4$) shifted the activation curve to the right (see D for quantification), without affecting the slope of the fit. The MR antagonist spironolactone (spiro, 100 nM, 5 min, $n = 6$) did not affect the activation of I_A, but prevented the corticosterone-induced shift. (D) A 5-min application of corticosterone (CORT) rapidly changed $V_{½}$ to −11 ± 3 mV ($n = 4$). The MR antagonist spironolactone alone (spiro, 100 nM, 5 min, $n = 6$) or intracellular application of the G protein inhibitor GDP-β-S (0.5 mM, 15 min, $n = 4$) did not affect $V_{½}$, but prevented the corticosterone-induced change in $V_{½}$. Error bars represent SEM. Data of all groups were subjected to ANOVA which revealed a significant treatment effect ($F = 3.6$, $P = 0.016$). *Post hoc* multiple comparison of the means (Tukey) showed that the shift in $V_{½}$ after CORT treatment differed significantly from the control as well as from the spiro + CORT and GDP-β-S + CORT groups; $*P < 0.05$, $**P < 0.01$.

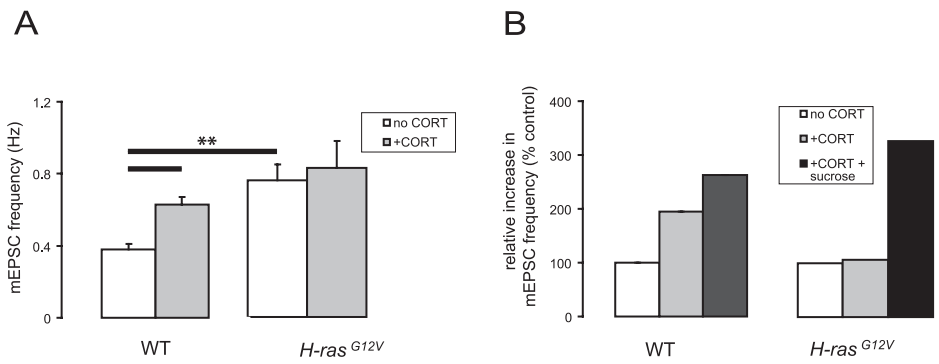

FIG. 5. mEPSC recordings from wild type and *H-ras*^{G12V}mice. (A) In the CA1 hippocampal area from transgenic *H-ras*^{G12V}. mice (H-ras; generated and maintained in a C57BL/6 background), the presynaptic H-Ras/ERK1/2 pathway is constitutively active. Bar graph shows the mean mEPSC frequency values under basal (vehicle) and corticosterone conditions. Compared to wild type (WT, $n = 6$), the basal mEPSC frequency was higher in the H-ras mice ($n = 5$). Application of 100 nM corticosterone (CORT) enhanced mEPSC frequency in wild type by 66%, but failed to do so in H-ras mutants (in both cases paired statistics), indicating that the pre- rather than postsynaptic ERK1/2 pathway mediated the effects of MR on mEPSC frequency. Error bars represent SEM; $^{**}P < 0.01$. (B) Sucrose (100 mM) enhanced mEPSC frequency in both WT and *H-ras*^{G12V}. mice to a similar extent (in percentage), as shown in these typical examples. In this case, mEPSC frequency is expressed as a percentage of the control value.

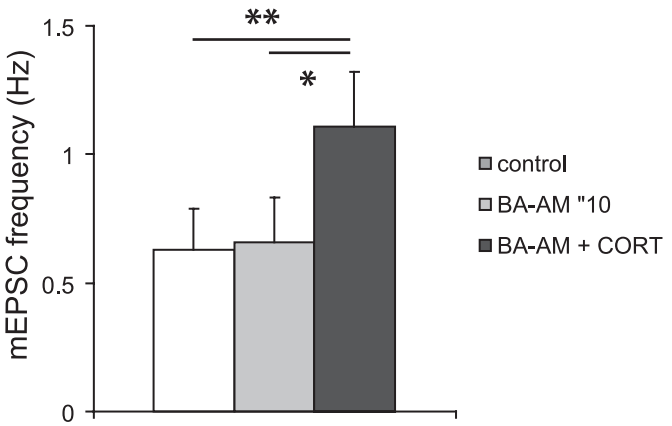

FIG. 6. Modulation of presynaptic intracellular calcium was not involved in the rapid effects of corticosterone. Properties of mEPSCs were recorded for 5 min under basal conditions (control, vehicle), followed by 10 min application of BAPTA-AM (BA-AM, 50 μM). Application of BAPTA-AM was continued for another 5 min but now in the presence of corticosterone (CORT, 100 nM). BAPTA-AM did not affect mEPSC frequency compared to baseline and failed to prevent the corticosterone-induced enhancement of mEPSC frequency (mean of $n = 5$). Error bars represent SEM; $^{*}P < 0.05$, $^{**}P < 0.01$. For all recordings, mEPSC amplitudes and kinetics remained unchanged (see Table 2).

the membrane-permeant calcium chelator BAPTA-AM (50 μM), which interferes with calcium-dependent neurotransmitter release in the hippocampus (see, e.g. Meyer *et al*., 2002; Hefft & Jonas, 2005). Application of BAPTA-AM (at least 10 min) did not affect mEPSC frequency, amplitude or kinetics (Fig. 6). In the continued presence of BAPTA-AM, 100 nM corticosterone enhanced mEPSC frequency significantly, by 78 ± 23% compared to baseline ($n = 5$, $P < 0.01$), excluding a role for corticosteroid-affected presynaptic calcium channels. Additional experiments with the nonselective calcium channel blocker cadmium chloride (50 μM) corroborated this finding (baseline, 0.53 ± 0.06 Hz; with Cd, 0.59 ± 0.05 Hz; with Cd and corticosterone, 0.93 ± 0.08 Hz; $n = 5$, $P \leq 0.05$).

Discussion

Stress exposure releases corticosterone from the adrenal glands. The hormone enters the brain, binds to nuclear receptors (which are particularly dense in the CA1 hippocampal area; see De Kloet *et al*., 2005), and as a consequence slowly and persistently alters the properties of pyramidal cells. This mostly involves a gene-mediated pathway, via GRs (De Kloet *et al*., 2005).

Recently, we discovered that CA1 cell mEPSC frequency is also rapidly and nongenomically affected by corticosterone, through MRs accessible from the extracellular site of the plasma membrane (Karst *et al*., 2005). Corticosterone, at concentrations reached during the stress induced peak in corticosteroid level (Linthorst *et al*., 1995), has been shown to increase the mEPSC frequency, due to an increased release probability for glutamate-containing vesicles (Karst *et al*., 2005). We here confirmed that in the hippocampus corticosterone markedly and rapidly increased mEPSC but not mIPSC frequency through the MR. We extended the notion that it is sufficient to access corticosteroid receptors from the extracellular domain by showing the it is in fact necessary to do so in order to evoke rapid nongenomic effects on the mEPSC frequency. The important implication could be that, at least in C57/Bl6 mice, the stress hormone corticosterone affects CA1 cell excitability over a wide time range, from minutes to hours after a single challenging situation. Some caution is necessary, as these data were all obtained under *in vitro* conditions; unfortunately, recording of rapid changes in mEPSC frequency under *in vivo* conditions is not yet technically possible.

To date, the signalling pathway underlying rapid effects via MRs in brain was entirely unknown. In view of earlier observations regarding rapid effects via the GR in the hypothalamus (Malcher-Lopes *et al*., 2006; Tasker *et al*., 2006) and oestrogen signalling pathways (Manavathi & Kumar, 2006), we hypothesized that corticosterone binds to postsynaptic membrane MRs, which activate the G protein-coupled ERK1/2 signalling pathway. Through a retrograde messenger this could lead to a change in presynaptic release probability.

Unexpectedly, the enhancement of the mEPSC frequency was found to occur independent of postsynaptic, G protein-coupled signalling pathways. This was supported by three observations. First, blockade of postsynaptic G proteins did not alter the effect of MR on mEPSC frequency; as a positive control we showed that a rapid effect of corticosterone on the postsynaptic I_A conductance was fully blocked in this paradigm (see further below). Second, if MRs mediating the effect on mEPSC frequency are located postsynaptically, a retrograde messenger would be necessary to accomplish the

presynaptic change in vesicle release probability. We examined the putative role of two well-known retrograde messengers, i.e. endocannabinoids and NO. Endocannabinoids have recently been implicated as retrograde messengers in rapid effects of corticosterone in the hypothalamus (Di *et al*., 2003; Malcher-Lopes *et al*., 2006). As the CB1 antagonist SR141617A failed to block the corticosterone effect on mEPSC frequency, the involvement of the CB1 receptor was excluded. Moreover (endo)cannabinoids, either via CB1 or other CB receptor types, suppress, rather than enhance, glutamate transmission (Hajos *et al*., 2001; Wilson *et al*., 2001; Hajos & Freund, 2002; Di *et al*., 2003; Kawamura *et al*., 2006; Takahashi & Castillo, 2006). This is in agreement with our observation that mEPSC frequency is slightly enhanced by SR141671A alone. A possible role for NO also seems unlikely as L-NAME, a NO synthase inhibitor, failed to block the corticosterone-induced enhancement of mEPSC frequency. One could reason that the duration of L-NAME application was insufficient to inhibit NO synthase activity. However, agonist-induced peaks in hippocampal NO synthesis can occur within 10 min (Ledo *et al*., 2005). Moreover, after 20 min (i.e., > 10 min of L-NAME alone plus 5–10 min of corticosterone) there was not even a trend towards suppression of mEPSC frequency. Although the putative role of other unknown retrograde messengers cannot be fully excluded, there is presently no support for the involvement of retrograde messengers in the corticosterone-induced mEPSC frequency enhancement.

The third observation incompatible with a postsynaptic signalling pathway relates to the experiments in the transgenic *H-Ras*^{G12V} mice. It is well known that ERK1/2 activation in the CA1 hippocampal region can take place in both the postsynaptic cell compartment and the presynaptic axon terminal (Yuan *et al*., 2002; Kushner *et al*., 2005). Unfortunately, we could not specifically inhibit the ERK1/2 signalling pathway pre- or postsynaptically with pharmacological tools, because all available ERK1/2 inhibitors are membrane-permeant. To solve this drawback, we made use of transgenic mice expressing a constitutively active form of H-ras (H-ras^{G12V}), which is abundantly present in axon terminals (Kushner *et al*., 2005) and shows enhanced phosphorylation of presynaptic ERK1/2 substrates but not postsynaptic ERK1/2 substrates. As presynaptic activation of the H-Ras–ERK1/2 pathway occluded the corticosterone-induced enhancement of mEPSC frequency, we propose that corticosterone enhances glutamate release probability via activation of the presynaptic ERK1/2 pathway. Therefore, contrary to our original hypothesis, the present experiments support the idea that corticosterone binds to MRs localized in the presynaptic membrane, which act as an upstream activator of the ERK1/2 signalling pathway. A critical role for ERK1/2 signalling is in line with an earlier report showing that corticosterone rapidly and nongenomically phosphorylates ERK1/2 although, in contrast to our electrophysiological observations, this effect was not sensitive to the MR antagonist spironolactone (Xiao *et al*., 2005). The exclusively presynaptic pathway in the hippocampus differs from the situation in the hypothalamus (Di *et al*., 2003), underlining the observation that signalling pathways for rapid corticosteroid effects in brain are regionally differentiated.

In addition to this presynaptic route, however, corticosterone can also rapidly affect CA1 cell properties through MRs inserted into the postsynaptic membrane. The modulation of I_A by corticosterone induced a shift to more depolarized potentials of the activation curve ($\sim$ 12 mV), resulting in a more depolarized $V_{½}$. This strongly resembles the effect seen after phosphorylation of the I_A channel via activation of the ERK1/2 pathway (Watanabe *et al*., 2002; Schrader *et al*., 2006). Furthermore, the corticosterone-mediated modulation of I_A could be prevented by inhibiting G protein activity using GDP-β-S, which is consistent with a G protein-activated (ERK1/2) pathway (Vanhoose *et al*., 2002; Berkeley & Levey, 2003). Unfortunately, as the MEK inhibitor U0126 directly affects the I_A channel independent of ERK1/2 or kinase activity (Yuan *et al*., 2006), we could not use this compound to test the involvement of MEK–ERK1/2. We hypothesize that the postsynaptic (membrane-located) MR is G protein-coupled and activates ERK1/2 to decrease I_A via phosphorylation of the potassium channel. The I_A channels serve to dampen excitatory input and modulate action potential back-propagation (Yuan *et al*., 2002). Therefore, the observed corticosterone-induced decrease in I_A could lead to an enhanced probability that excitatory input results in action potential generation.

The apparent difference in receptor location for the two effects and their equally rapid onset make it plausible that both the enhancement of glutamate transmission and the decrease in postsynaptic I_A occur simultaneously or within a short interval of each other. If so, presynaptic membrane-located MRs could facilitate excitatory neurotransmission shortly after corticosterone levels rise (e.g. stress); simultaneously, through postsynaptic membrane MRs that inhibit the postsynaptic I_A conductance, the chances that enhanced excitatory input leads to changes in CA1 pyramidal neuron activity increase provided that other synaptic components remain similar. We propose that in this way MRs located in the pre- as well as postsynaptic membrane can rapidly enhance CA1 excitability shortly (< 5 min) after corticosterone levels start to rise. Clearly, it will be also important in future to test these assumptions, based on *in vitro* effects of corticosterone, *in vivo*, e.g. after stress. In support, though, rapid increases in extracellular glutamate level upon corticosterone administration have been demonstrated with microdialysis in freely moving animals (Venero & Borrell, 1999). If so, the dual possibility of rapidly enhancing hippocampal CA1 cell excitability with corticosterone underlines the unique potential of this hormone during the stress response: shortly after stress exposure hippocampal excitability might be raised through multiple pathways involving membrane-located MRs. Concomitantly, a slow gene-mediated pathway involving nuclear GRs is started and this, several hours after stress exposure, will normalize hippocampal activity.

Acknowledgements

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Abbreviations

aCSF, artificial cerebral spinal fluid; BAPTA-AM, 1,2-bis-(o-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid, tetraacetoxymethyl ester; ERK, extracellular signal-regulated kinase; GDP, guanosine diphosphate; GR, glucocorticoid receptor; GTP, guanosine triphosphate; I_A, A-current; L-NAME, N(G)-nitro-L-arginine methyl ester; MEK, mitogen-activated protein kinase/ERK kinase; mEPSC, miniature excitatory postsynaptic current; mIPSC, miniature inhibitory postsynaptic current; MR, mineralocorticoid receptor; TPA, phorbol 12-myristate 13-acetate.

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