Protein kinase C epsilon mediation of CRF- and ethanol-induced GABA release in central amygdala

Michal Bajo*†, Maureen T. Cruz†‡, George R. Siggins*, Robert Messing§, and Marisa Roberto‡¶‖

*Molecular and Integrative Neurosciences Department, ‡Committee on Neurobiology of Addictive Disorders, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037; §Ernest Gallo Clinic and Research Center, Department of Neurology, University of California San Francisco, Emeryville, CA 94608; and ¶Pearson Center for Alcoholism and Addiction Research, 10550 North Torrey Pines Road, La Jolla, CA 92037

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In the central amygdala (CeA), ethanol acts via corticotrophin-releasing factor (CRF) type 1 receptors to enhance GABA release. Amygdala CRF mediates anxiety associated with stress and drug dependence, and it regulates ethanol intake. Because mutant mice that lack PKCε exhibit reduced anxiety-like behavior and alcohol consumption, we investigated whether PKCε lies downstream of CRF_1 receptors in the CeA. Compared with $PKC\varepsilon^{+/+}$ CeA neurons, $PKC\varepsilon^{-/-}$ neurons showed increased GABAergic tone due to enhanced GABA release. CRF and ethanol stimulated GABA release in the $PKC\varepsilon^{+/+}$ CeA, but not in the $PKC\varepsilon^{-/-}$ CeA. A PKCε-specific inhibitor blocked both CRF- and ethanol-induced GABA release in the $PKC\varepsilon^{+/+}$ CeA, confirming findings in the $PKC\varepsilon^{-/-}$ CeA. These results identify a PKCε signaling pathway in the CeA that is activated by CRF_1 receptor stimulation, mediates GABA release at nerve terminals, and regulates anxiety and alcohol consumption.

GABA transmission | alcohol | electrophysiology | anxiety | presynaptic transmission

The neuropeptide corticotrophin-releasing factor (CRF) contributes to stress-related disorders such as anxiety and depression. Elevated levels of CRF are commonly found in the cerebrospinal fluid of patients suffering from depression or anxiety disorders (1, 2). Moreover, intracerebroventricular infusion of CRF or transgenic overexpression of CRF increases anxiety-like behavior in rodents (3, 4). The anxiogenic effects of CRF are mediated by type 1 CRF receptors (CRF_1Rs), as demonstrated in CRF_1R-deficient mice, which show reduced anxiety-like behavior (5, 6). CRF_1Rs are abundantly expressed in the cortex, cerebellum, hippocampus, amygdala, olfactory bulb, and pituitary (7–9). Of particular interest is the amygdala, a pivotal region in the acquisition and expression of fear conditioning (10). In the rat, intra-amygdalar injections of CRF increase inhibitory avoidance responses while reducing exploratory behavior in an open field (11). In addition, injection of CRF antagonists and CRF_1R antisense oligonucleotides into the rat amygdala reduces stress-induced, anxiety-like behavior (12, 13). Thus, CRF expression in the amygdala is especially critical in the regulation of anxiety.

Prolonged alcohol dependence produces a negative emotional state characterized by heightened anxiety and reactivity to stress, which increases alcohol drinking, probably in an attempt to relieve these negative symptoms (14). Recent evidence suggests that this negative affective state is regulated by extrahypothalamic CRF. CRF release in the central amygdala (CeA) and bed nucleus of the stria terminalis (BNST) is increased in alcohol-dependent animals (15, 16) and appears to contribute to alcohol withdrawal-related anxiety, which can be reduced by CRF receptor antagonists injected into the CeA (17). CRF also contributes to increased alcohol consumption in dependent animals because their increased ethanol self-administration is reduced by CRF_1 receptor antagonists (18) or the deletion of the CRF_1R (19).

CRF is abundant in the CeA, where it is coexpressed with GABA (20), suggesting that CRF may modulate GABAergic signaling within the amygdala. Most of the CeA neurons in rodents are GABAergic inhibitory neurons with inhibitory recurrent or feed-forward connections, as well as inhibitory projections to brainstem nuclei (see Fig. 1) (21, 22). We previously showed that CRF and ethanol enhance GABA release from mouse CeA neurons in a CRF_1R-dependent manner (23). This finding suggested a cellular mechanism by which CRF could modulate the behavioral and motivational effects of ethanol. CRF acting at CRF_1Rs may also regulate alcohol drinking in humans because variants in the CRHR1 gene encoding the human CRF_1R have recently been associated with binge drinking and high alcohol intake in two independent sample populations (24).

It is not known how activation of CRF_1Rs in the amygdala influence anxiety and alcohol drinking. However, recent *in vitro* evidence indicates that PKC signaling is stimulated by CRF_1R activation (25, 26). PKC is a family of serine-threonine kinases that respond to lipid second messengers and have been implicated in neurobehavioral disorders, including anxiety and drug abuse (27). Among the PKC isozymes, we hypothesized that PKCε mediates downstream effects of CRF_1R activation in the CeA because PKCε is expressed throughout the amygdala (28) and $PKC\varepsilon^{-/-}$ mice show reduced anxiety-like behavior (29) and reduced alcohol consumption (30, 31). To examine this hypothesis, we studied the role of PKCε signaling in basal CeA GABAergic transmission and in ethanol- and CRF-induced GABA release in an *in vitro* slice preparation using both genetic and pharmacological approaches. Our findings indicate that PKCε is a key regulator of basal and of CRF- and ethanol-stimulated GABA release from CeA neurons.

Results

Basal GABAergic Transmission Is Enhanced in CeA Neurons from $PKC\varepsilon^{-/-}$ Mice. We recorded from 118 CeA neurons with a mean resting membrane potential of -76 ± 2 mV and a mean input resistance of 106 ± 4 MΩ. $PKC\varepsilon^{-/-}$ and $PKC\varepsilon^{+/+}$ littermates did not show significant differences in these properties. We evoked pharmacologically isolated $GABA_A$ receptor-mediated IPSPs ($GABA_A$ IPSPs) by stimulating locally within the CeA. Interestingly, baseline IPSP input–output (I–O) curves generated by equivalent stimulus intensities were higher in CeA neurons from $PKC\varepsilon^{-/-}$ mice compared with those from littermates (Fig. 2*A*). To test whether this synaptic enhancement could derive from a presynaptic site of action, we examined paired-pulse facilitation (PPF) of the IPSPs at 50-, 100-, and 200-msec interstimulus intervals (ISIs). Generally, changes in PPF are inversely related

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†M.B. and M.T.C. contributed equally to this work.

‖To whom correspondence should be addressed. E-mail: mroberto@scripps.edu.

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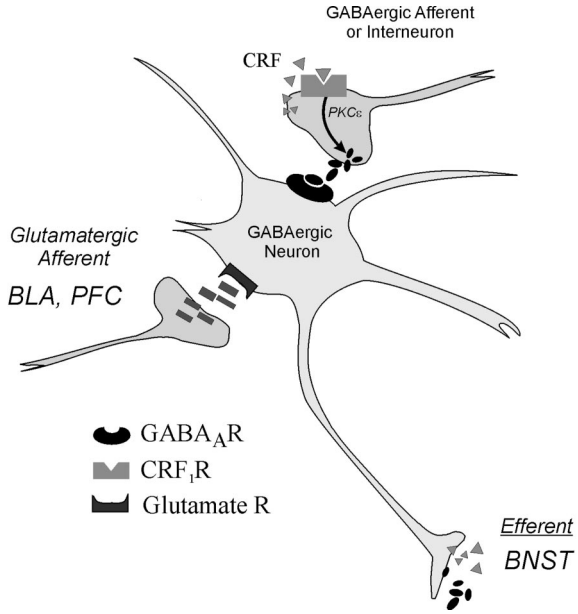

Fig. 1. Simplified schematic of rodent CeA circuitry and hypothetical sites of ethanol and CRF action on GABAergic synapses. Most neurons in the CeA are GABAergic inhibitory projection or interneurons that contain CRF or other neuropeptides as cotransmitters. (Upper synapse) Ethanol may enhance the release of GABA (filled ellipsoids) from GABAergic afferents or interneurons either via the release from the same terminal as CRF (gray triangles), which then acts on CRF1 receptors on the terminal to elicit (black arrow) release of more GABA via a PKCε-mediated mechanism, or direct activation of CRF1 receptors to elicit the release of more GABA. Thus, CRF and ethanol both augment the inhibition of CeA projection interneurons (cocontaining CRF, opioids, or NPY), leading to excitation of downstream (e.g., BNST) neurons by disinhibition. Activation of presynaptic opioid, CB1, or NPY receptors (data not shown) may reduce GABA release onto CeA inhibitory projection neurons, increasing their excitability and release of GABA onto downstream targets such as the BNST. (Lower synapse) Glutamatergic afferents from basolateral amygdala (BLA) or prefrontal cortex (PFC) excite the CeA inhibitory neurons via release of glutamate (filled rectangles) and activation of glutamate receptors.

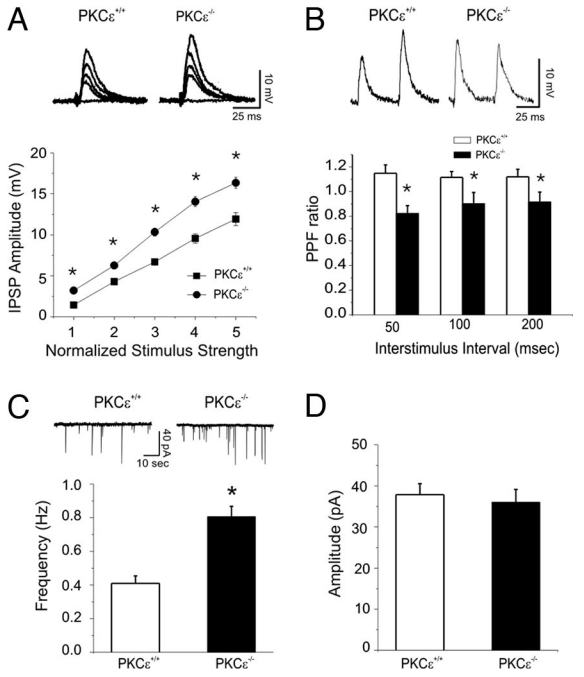

Fig. 2. Basal GABAergic transmission is enhanced in CeA of PKCε$^{-/-}$ mice. (*A*) (*Upper*) Superimposed traces of five representative GABA$_A$ IPSPs evoked by five incrementally increasing stimulus intensities in slices from PKCε$^{+/+}$ (*Left*) and PKCε$^{-/-}$ (*Right*) mice. (*Lower*) The mean baseline IPSP amplitudes were significantly increased (*, $P < 0.05$) in PKCε$^{-/-}$ CeA neurons ($n = 31$) compared with PKCε$^{+/+}$ neurons ($n = 28$). (*B*) Baseline PPF of IPSPs is decreased in CeA from PKCε$^{-/-}$ mice. (*Upper*) Representative traces of a paired-pulse study (at 50-msec ISI) of IPSPs in CeA neurons from PKCε$^{+/+}$ (*Left*) and PKCε$^{-/-}$ (*Right*) mice. (*Lower*) Baseline PPF of IPSPs was significantly (*, $P < 0.001$) reduced in CeA from PKCε$^{-/-}$ mice ($n = 25$) compared with PKCε$^{+/+}$ mice ($n = 26$). (*C*) (*Upper*) Representative mIPSCs from PKCε$^{+/+}$ (*Left*; $n = 15$) and PKCε$^{-/-}$ (*Right*; $n = 9$). (*Lower*) The mean frequency of mIPSCs was significantly (*, $P < 0.001$) greater in CeA neurons from PKCε$^{-/-}$ mice ($n = 8$) compared with those of PKCε$^{+/+}$ mice ($n = 8$). (*D*) The same group of neurons showed no significant alteration of mean amplitude of mIPSCs in PKCε$^{-/-}$ mice. Statistical significance (*, $P < 0.05$) was calculated by two-tailed *t* tests.

to transmitter release (32, 33). When compared with the ratio from littermate wild-type controls (Fig. 2*B*), we found that the baseline PPF ratio of IPSPs was decreased in CeA neurons from PKCε$^{-/-}$ mice, suggesting that GABA release was augmented in the CeA of PKCε$^{-/-}$ mice.

To further characterize the increased GABA release in PKCε$^{-/-}$ mice, we recorded pharmacologically isolated spontaneous miniature GABA$_A$ IPSCs (mIPSCs) using whole-cell patch clamp in the presence of 1 μM TTX to eliminate action potential-dependent neurotransmitter release. Notably, the mean baseline frequency of mIPSCs was greater in CeA neurons from PKCε$^{-/-}$ mice compared with neurons from PKCε$^{+/+}$ littermates (Fig. 2*C*), suggesting increased basal GABA release in the CeA of PKCε$^{-/-}$ mice. In contrast, the mean amplitude of mIPSCs was similar in PKCε$^{-/-}$ and PKCε$^{+/+}$ CeA neurons (Fig. 2*D*), indicating no significant difference between the two genotypes in postsynaptic GABA$_A$ receptor activation by spontaneously released GABA. Also, we did not observe differences in decay time or rise time of basal mIPSCs in CeA neurons from PKCε$^{-/-}$ and PKCε$^{+/+}$ mice (data not shown).

CRF Enhancement of GABAergic Transmission Is Blocked in CeA of PKCε$^{-/-}$ Mice. We previously reported that CRF augments GABAergic transmission in CeA slices from C57BL/6J mice via activation of CRF$_1$Rs (23). Because new data suggest that CRF$_1$R activation may stimulate a PKC pathway (25, 26), we explored the possible role of PKCε in CRF stimulation of GABA release in the CeA. As we reported for C57BL/6J mice (23), superfusion of CeA slices from PKCε$^{+/+}$ mice with 200 nM CRF for 10 min increased the mean amplitude of evoked IPSPs by 45% (Fig. 3*A*). To test whether the site of CRF action was pre- or postsynaptic, we examined the effect of CRF on PPF of the IPSPs at 50-, 100-, and 200-msec ISIs and found that CRF decreased the PPF of IPSPs (Fig. 3*B*) at 50- and 100-msec ISIs, suggesting that CRF acts presynaptically to increase GABA release in the mouse CeA. We then examined neurons from PKCε$^{-/-}$ mice to investigate whether PKCε is required for CRF-stimulated GABA release. Unlike CeA neurons from PKCε$^{+/+}$ mice, CRF-induced enhancement of evoked IPSP amplitude was absent in neurons from PKCε$^{-/-}$ mice (Fig. 3*C*). In addition, CRF did not alter the PPF ratio of IPSPs at any of the three ISIs tested (Fig. 3*D*).

To further verify the presynaptic site of CRF action, we also evaluated the effect of CRF on mIPSCs. Superfusion of 200 nM CRF onto PKCε$^{+/+}$ CeA neurons increased the mean frequency of mIPSCs and shifted the cumulative frequency distribution to shorter interevent intervals (Fig. 4 *A* and *E*), supporting the PPF data indicating increased presynaptic release of GABA by CRF. These mIPSCs were completely blocked by superfusion of bicuculline (data not shown), indicating their mediation by GABA$_A$ receptors. CRF did not significantly alter mIPSC amplitudes (Fig. 4*C*). Notably, in contrast to CeA neurons of

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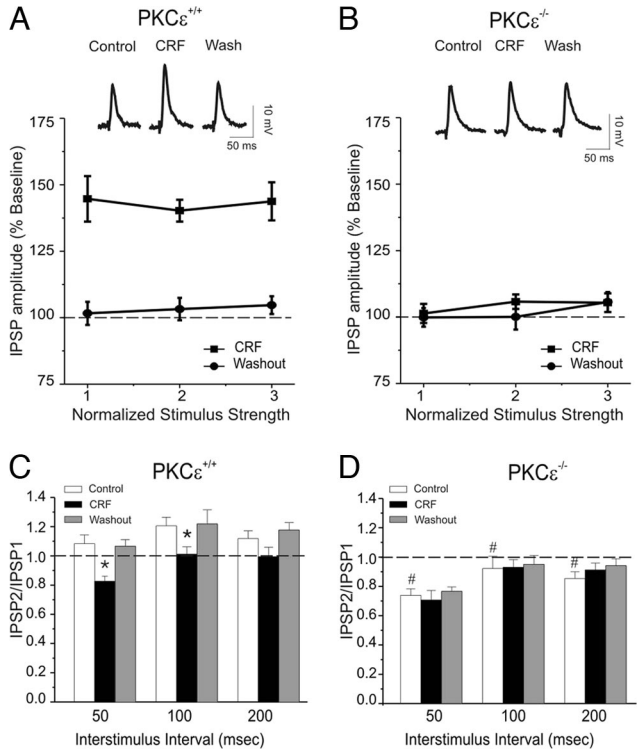

Fig. 3. CRF increases GABAergic transmission in CeA neurons from PKC$\varepsilon^{+/+}$ mice, but not from PKC$\varepsilon^{-/-}$ mice. (*A*) (*Upper*) Representative $GABA_A$ IPSPs in a CeA slice from a PKC$\varepsilon^{+/+}$ mouse recorded before, during, and after superfusion of 200 nM CRF. (*Lower*) CRF increased the mean IPSP amplitudes in PKC$\varepsilon^{+/+}$ CeA neurons ($n = 12$; *, $P < 0.001$) with recovery on washout (20 min). (*B*) CRF reduced the PPF ratio of IPSPs in PKC$\varepsilon^{+/+}$ neurons ($n = 12$; *, $P < 0.05$). (*C*) (*Upper*) IPSPs in a CeA slice from a PKC$\varepsilon^{-/-}$ mouse. (*Lower*) CRF did not alter the mean IPSP amplitudes in PKC$\varepsilon^{-/-}$ CeA neurons ($n = 13$). (*D*) CRF did not alter PPF ratios of IPSPs in CeA PKC$\varepsilon^{-/-}$ neurons ($n = 13$). Statistical significance (#, $P < 0.05$) was calculated by repeated measures ANOVA and Newman–Keuls tests.

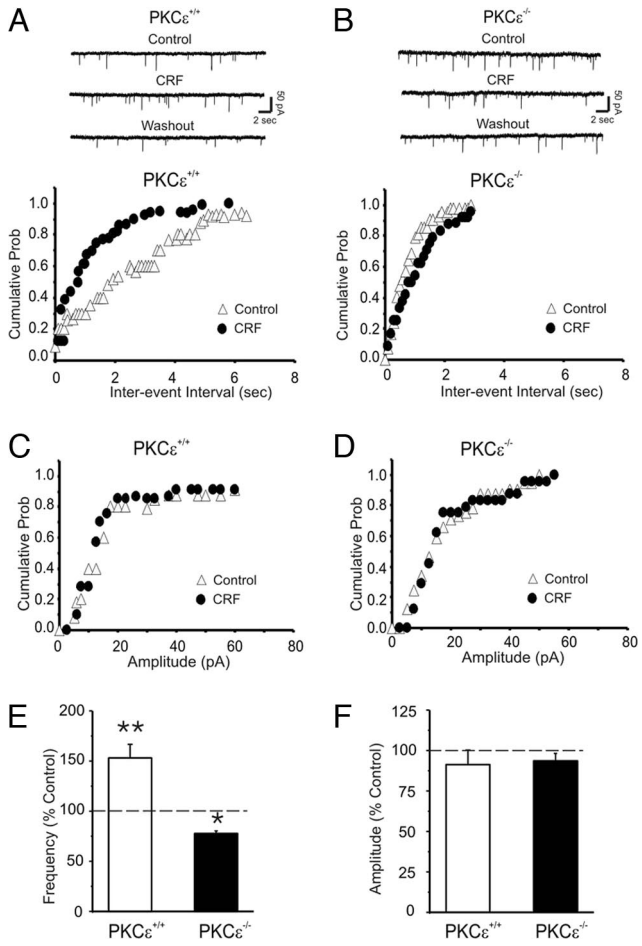

Fig. 4. CRF increases GABAergic transmission in CeA through a presynaptic, PKCε-dependent mechanism. (*A*) (*Upper*) Representative mIPSCs from a PKC$\varepsilon^{+/+}$ CeA neuron. CRF increased (*, $P < 0.05$) the frequency but not the amplitude of the mIPSCs. (*Lower*) Cumulative frequency histogram from the same PKC$\varepsilon^{+/+}$ neuron indicating shorter interevent intervals (higher frequency) during application of CRF. (*B*) (*Upper*) Representative mIPSCs from a PKC$\varepsilon^{-/-}$ CeA neuron. CRF significantly (*, $P < 0.05$) decreased the frequency of the mIPSCs. (*Lower*) Cumulative frequency histogram from the PKC$\varepsilon^{-/-}$ mouse neuron, indicating longer interevent intervals (lower frequency) during the application of CRF. (*C* and *D*) Cumulative amplitude histogram from the PKC$\varepsilon^{+/+}$ (*C*) and the PKC$\varepsilon^{-/-}$ CeA neuron (*D*), showing no ethanol-induced alteration in the distribution of mIPSC amplitudes. (*E*) CRF increased the mean frequency (expressed as percentage of control) of mIPSCs in PKC$\varepsilon^{+/+}$ CeA neurons ($n = 5$), but decreased it in PKC$\varepsilon^{-/-}$ neurons ($n = 3$), with recovery on washout (data not shown). **, $P < 0.0001$ and *, $P < 0.005$ compared with a baseline mean frequency of 100% (dashed line) by one sample *t* tests. (*F*) CRF did not alter the mean mIPSC amplitudes in either genotype.

PKC$\varepsilon^{+/+}$ mice, CRF decreased mean mIPSC frequency in neurons from PKC$\varepsilon^{-/-}$ mice (Fig. 4 *B* and *E*), suggesting that CRF decreases vesicular GABA release in PKC$\varepsilon^{-/-}$ neurons. As in the CeA of PKC$\varepsilon^{+/+}$ mice, CRF did not affect the mean amplitude of mIPSCs in the CeA of PKC$\varepsilon^{-/-}$ mice (Fig. 4 *D* and *F*), again indicating the lack of postsynaptic CRF effects on $GABA_A$ receptor stimulation by GABA in the mouse CeA.

Because developmental changes or compensatory effects of other gene products may confound studies in gene-targeted mice, we pharmacologically confirmed the role of PKCε in regulating GABA release from CeA neurons by using a PKCε inhibitor peptide, Tat-εV1–2 (34). Superfusion of the inhibitor alone (500 nM) onto slices from PKC$\varepsilon^{+/+}$ mice increased the mean evoked IPSP amplitude and decreased the PPF of IPSPs in CeA neurons [supporting information (SI) Fig. S1 *A* and *B*]. These results suggest a constitutive role for PKCε in tonically inhibiting GABA release at CeA synapses that may account, in part, for increased basal GABAergic transmission seen in PKC$\varepsilon^{-/-}$ mice. Moreover, pretreatment of CeA neurons with the Tat-εV1–2 peptide blocked the CRF-induced augmentation of the evoked IPSP amplitudes (Fig. S1 *B* and *C*) and prevented the CRF-induced decrease in PPF of IPSPs (Fig. S1*D*). These results resemble those obtained in CeA neurons from PKC$\varepsilon^{-/-}$ mice (Fig. 3 *C* and *D*) and support the conclusion that PKCε mediates CRF activation of GABA release.

Ethanol Enhancement of GABAergic Transmission Is Blocked in CeA from PKC$\varepsilon^{-/-}$ Mice. Previously, we found that ethanol dose-dependently increased GABAergic transmission in CeA slices by increasing GABA release through a mechanism dependent on the activation of CRF_1Rs (23, 33). Having determined that CRF-induced GABA release is regulated by PKCε, we investigated whether ethanol-stimulated GABA release also involves PKCε. In CeA slices from PKC$\varepsilon^{+/+}$ mice, 10-min superfusion of 44 mM ethanol increased the mean amplitude of evoked IPSPs by 47% at each stimulus strength tested ($n = 19$) (Fig. 5*A*). Ethanol also significantly decreased the PPF of IPSPs (Fig. 5*B*) at each ISI, suggesting that ethanol increases GABA release. Next, we examined neurons from PKC$\varepsilon^{-/-}$ mice to investigate whether PKCε is required for ethanol-stimulated GABA release. Notably, in contrast to PKC$\varepsilon^{+/+}$ neurons (Fig. 5 *A* and *B*), the ethanol-induced augmentation of evoked IPSP amplitudes was completely absent in CeA neurons from PKC$\varepsilon^{-/-}$ mice (Fig. 5*C*), as was the ethanol-induced reduction in PPF of IPSPs (Fig. 5*D*).

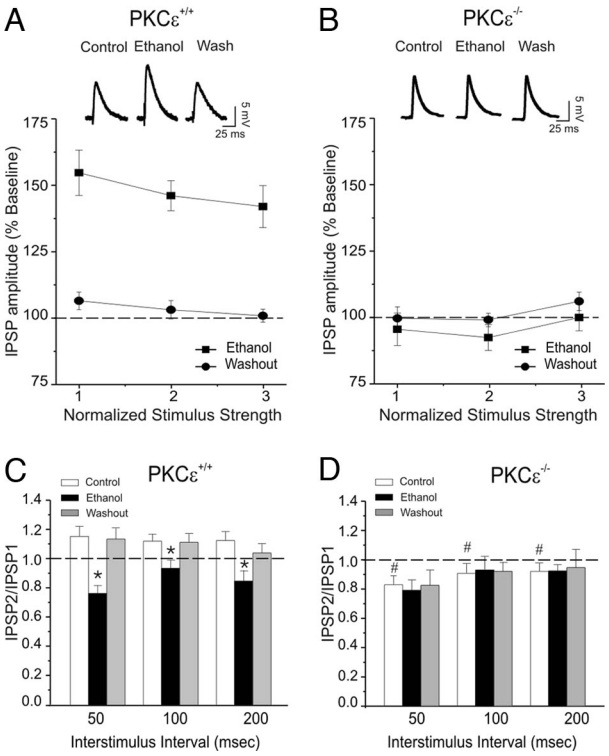

Fig. 5. Ethanol increases GABAergic transmission in CeA neurons from PKCε$^{+/+}$ mice, but not from PKCε$^{-/-}$ mice. (*A*) (*Upper*) Representative IPSPs in a PKCε$^{+/+}$ CeA neuron before, during, and after superfusion of 44 mM ethanol. (*Lower*) Ethanol significantly increased (*, $P < 0.001$) the mean IPSPs in PKCε$^{+/+}$ CeA neurons ($n = 19$). (*B*) Ethanol-reduced PPF ratios of IPSPs (*, $P < 0.001$) with recovery on washout in PKCε$^{+/+}$ CeA neurons. (*C*) (*Upper*) IPSPs in a CeA neuron from a PKCε$^{-/-}$ mouse. (*Lower*) Ethanol did not alter the mean IPSP amplitudes in 15 CeA neurons from PKCε$^{-/-}$ mice. (*D*) Ethanol did not alter the ratios at any of the ISIs tested in PKCε$^{-/-}$ CeA neurons. Statistical significance (#, $P < 0.05$) was calculated by repeated measures ANOVA and post hoc Newman–Keuls tests.

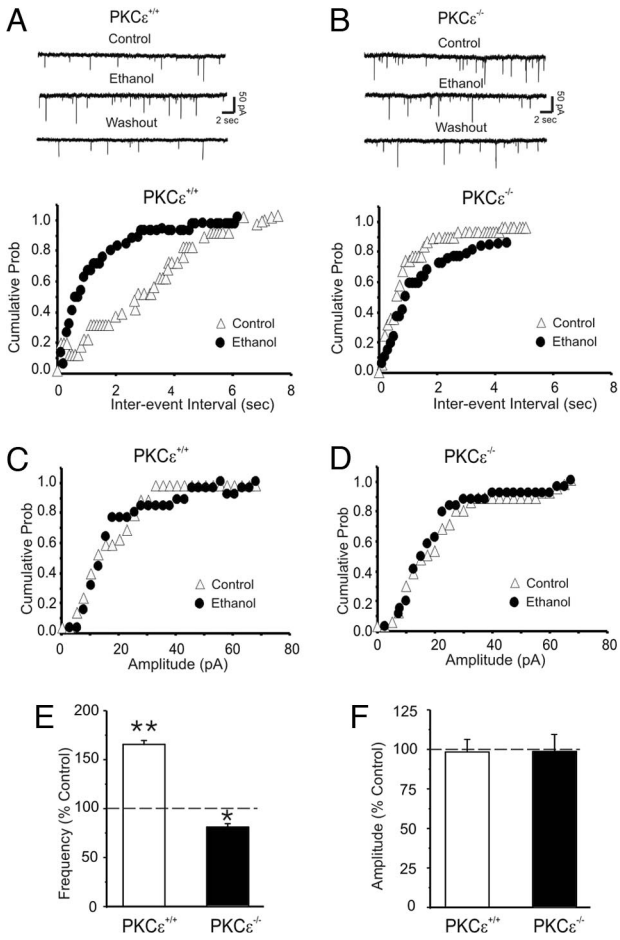

Fig. 6. Ethanol, like CRF (see Fig. 4), increases GABAergic transmission in the CeA of PKCε$^{+/+}$, but not in PKCε$^{-/-}$ mice. (*A*) (*Upper*) mIPSCs from a PKCε$^{+/+}$ CeA neuron. Ethanol increased the frequency but not the amplitude of the mIPSCs. (*Lower*) Cumulative frequency histogram from the same PKCε$^{+/+}$ neuron, indicating shorter interevent intervals (higher frequency) during ethanol superfusion. (*B*) (*Upper*) mIPSCs from a PKCε$^{-/-}$ CeA neuron. Ethanol significantly decreased the frequency of the mIPSCs. (*Lower*) Cumulative frequency histogram from this PKCε$^{-/-}$ neuron, indicating longer interevent intervals (lower frequency) during ethanol application. (*C* and *D*) Cumulative amplitude histogram from the PKCε$^{+/+}$ (*C*) and the PKCε$^{-/-}$ CeA neuron (*D*), showing no ethanol-induced alteration in the distribution of mIPSC amplitudes. (*E*) Ethanol significantly increased the mean frequency of mIPSCs in 6 PKCε$^{+/+}$ CeA neurons but decreased it in 6 CeA PKCε$^{-/-}$ neurons, with recovery on washout (data not shown). **, $P < 0.0001$ and *, $P < 0.005$ compared with a baseline mean frequency of 100% (dashed line) by one sample *t* tests. (*F*) Ethanol did not alter the mean mIPSC amplitudes in either genotype.

To further verify the presynaptic site of ethanol action, we also evaluated the effect of ethanol on mIPSCs in the CeA. Ethanol, like CRF, increased the mean frequency of mIPSCs and shifted the cumulative frequency distribution to shorter interevent intervals in PKCe$^{+/+}$ CeA neurons (Fig. 6 *A* and *E*), supporting PPF data indicating an increased release of GABA by ethanol. Ethanol, like CRF, did not significantly alter the mIPSC amplitudes (Fig. 6 *C* and *F*). In sharp contrast to CeA neurons of PKCε$^{+/+}$ littermates, ethanol significantly ($P < 0.05$, two-tailed *t* test) decreased the mean mIPSC frequency in CeA neurons from PKCε$^{-/-}$ mice (Fig. 6 *B* and *E*), suggesting that ethanol actually decreases vesicular GABA release in these neurons. As in PKCε$^{+/+}$ neurons, ethanol did not affect the mean amplitude of mIPSCs in PKCε$^{-/-}$ neurons (Fig. 6 *D* and *F*), again indicating lack of postsynaptic ethanol effects on $GABA_A$ receptor activation by synaptically released GABA in the mouse CeA.

To further demonstrate the involvement of PKCε in ethanol effects on CeA neurons, we superfused the Tat-εV1–2 peptide (500 nM) onto another group of PKCε$^{+/+}$ CeA neurons for 30 min before superfusion of ethanol in the continued presence of Tat-εV1–2. Pretreatment with the Tat-εV1–2 peptide completely abolished the ethanol-induced increase in IPSP amplitudes (Fig. S1 *B* and *C*). Instead, ethanol actually significantly decreased ($P < 0.05$) the evoked IPSP amplitudes in the presence of the PKCε inhibitor. In addition, the Tat-εV1–2 peptide blocked the ethanol-induced decrease in PPF of IPSPs (Fig. S1*D*). These results confirm that PKCε mediates ethanol enhancement of evoked GABA release in the CeA.

Discussion

Recent evidence indicates that PKC can be activated by CRF signaling in the nervous system. PKC contributes to CRF-mediated long-term depression of climbing fiber-parallel fiber synapses in the cerebellum (35) and to CRF-stimulated neuronal activity in hippocampal slices from BALB/c mice (36). In AtT-20 cells, CRF acting at CRF_1Rs increases CREB phosphorylation through a PKC-dependent mechanism (25), whereas in MN9D dopaminergic cells, CRF_1R activation produces a PKC-dependent inhibition of T-type calcium channels (26). However, the PKC isozymes that mediate these CRF responses are not known. Here we demonstrate a specific role for the PKCε in CRF-stimulated GABA release from neurons of the CeA.

Moreover, consistent with our previous observation that ethanol-induced GABA release in the amygdala is CRF_1R-dependent (23), here we also find that ethanol-stimulated vesicular GABA release depends on PKCε. Taken together, these findings indicate a signaling pathway whereby CRF, acting at presynaptic CRF_1Rs in the amygdala, activates PKCε to stimulate GABA release. Because CRF is anxiogenic and plays an important role in promoting alcohol drinking (14), disturbance of this CRF_1R–PKCε signaling pathway in the CeA likely contributes to decreased anxiety-like behavior and decreased alcohol consumption in $PKC\varepsilon^{-/-}$ mice.

Without drug treatment, evoked $GABA_A$ IPSPs were larger, paired-pulse facilitation of IPSPs was reduced, and the frequency of mIPSCs was higher in $PKC\varepsilon^{-/-}$ neurons when compared with $PKC\varepsilon^{+/+}$ neurons. This finding suggests a basal level of CRF_1R activation or PKCε activity in wild-type neurons that serves to limit spontaneous GABA release. Both CRF and ethanol increased evoked IPSP amplitudes, decreased paired-pulse facilitation of IPSPs, and increased the frequency of spontaneous mIPSCs in $PKC\varepsilon^{+/+}$ neurons, but not in $PKC\varepsilon^{-/-}$ neurons, indicating that under drug-stimulated conditions PKCε facilitates vesicular GABA release. The inability of ethanol and CRF to stimulate release in $PKC\varepsilon^{-/-}$ mice was not due to a ceiling effect because both agents actually diminished evoked IPSP amplitudes in the presence of a PKCe inhibitor by 5–10% and decreased the frequency of spontaneous mIPSCs in $PKC\varepsilon^{-/-}$ neurons by ≈25% from baseline. Therefore, these results indicate that PKCε serves two roles in the CeA: (*i*) reduce spontaneous baseline GABA release, and (*ii*) mediate CRF- and ethanol-stimulated release of GABA.

As schematized in Fig. 1, most of the CeA neurons are GABAergic with inhibitory projections to brainstem nuclei. Because GABAergic drugs are typically robust anxiolytics (37), it may seem paradoxical that CRF would augment GABAergic transmission in a brain region known to be involved in stress-related behavior. However, we find that anxiolytic agents like nociceptin (38), endocannabinoids, and NPY diminish IPSPs in CeA neurons, whereas anxiogenic agents such as CRF (23) and galanin augment IPSPs in CeA neurons. Therefore, CRF_1Rs and their linkage to PKCε may modulate inhibitory CeA gating that regulates information flow through intra-amygdala circuits. By decreasing GABA release at nerve terminals of amygdala projection neurons, this signaling pathway could decrease the inhibition of downstream targets such as the BNST, thereby disinhibiting (i.e., exciting) these areas. Thus, the dogma of an inverted relationship between GABA and anxiety may apply to GABA's effects in these target areas, but not to its actions within the CeA.

It is important to note that our present findings represent only a subset of the actions of CRF and ethanol in the brain. In contrast to its presynaptic effects, we did not observe a direct postsynaptic effect of CRF or ethanol on membrane properties of CeA neurons. Because CRF and ethanol only increased evoked IPSP, but not mIPSC amplitudes, we can conclude that there is no direct effect of CRF or ethanol on $GABA_A$ receptors in the CeA per se.

Ethanol is anxiolytic when administered systemically, whereas amygdala CRF promotes anxiety-like behavior (14). Given the parallel actions of ethanol and CRF in promoting GABA release within the CeA, these opposite behavioral responses may appear paradoxical. However, with respect to the CeA neuronal population we have studied, ethanol has more than one effect: It enhances GABA release, but also postsynaptically reduces NMDAR- and non-NMDAR-mediated glutamatergic excitatory postsynaptic potentials (39), which could contribute to the anxiolytic effects of systemic ethanol. Additionally, in contrast to endogenous CRF, which is localized to specific brain circuits, systemic ethanol has a more global effect in enhancing GABAergic signaling in other brain regions involved in anxiety, such as those downstream of the CeA like the BNST, which also could explain the net anxiolytic effect of systemic ethanol (Fig. 1).

In summary, a complete understanding of the cellular and molecular underpinnings of anxiety and drug abuse may require a region-by-region exploration of the many neuronal circuits shown to be involved in these behaviors. Nonetheless, our present findings indicate that PKCε acting downstream of the CRF_1R plays a pivotal role in the actions of two agents, CRF and ethanol, which are known to affect the GABA system in a brain region critically involved in stress-related behaviors such as anxiety and substance abuse.

Materials and Methods

Mouse Breeding and Care. We used $PKC\varepsilon^{-/-}$ mice previously generated by homologous recombination in J1 ES cells (40). We conducted all mouse breeding and care procedures in accordance with the Ernest Gallo Clinic and Research Center and The Scripps Research Institute Institutional Animal Care and Use Committee (IACUC) policies.

Slice Preparation. We prepared CeA slices as previously described (23, 33) from male $PKC\varepsilon^{-/-}$ and $PKC\varepsilon^{+/+}$ littermates (5–6 months old at the time of experimentation). All electrophysiological experiments were performed in accordance with The Scripps Research Institute IACUC and National Institutes of Health guidelines on the care and use of laboratory animals. We cut 400-μm-thick transverse slices on a vibrating microtome (Vibratome Series 3000; Technical Products International), incubated them in an interface configuration for 30 min, and completely submerged and continuously superfused (flow rate of 2–4 ml/min) them with warm (31°C), gassed artificial cerebrospinal fluid (aCSF) of the following composition: 130 mM NaCl, 3.5 mM KCl, 1.25 mM NaH_2PO_4, 1.5 mM $MgSO_4{\cdot}7H_2O$, 2.0 mM $CaCl_2$, 24 mM $NaHCO_3$, and 10 mM glucose. Drugs were added to the aCSF from stock solutions to obtain known concentrations in the superfusate.

Electrophysiology of Evoked Responses. For studies of evoked IPSPs, we recorded from CeA neurons with sharp micropipettes (3 M KCl) in current-clamp mode. We held most neurons near their resting membrane potential. We evoked pharmacologically isolated $GABA_A$ IPSPs by stimulating locally within the CeA through a bipolar stimulating electrode while superfusing the slices with the glutamate receptor blockers 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) and DL-2-amino-5-phosphonovalerate (APV; 30 μM) and the $GABA_B$ receptor antagonist CGP 55845A (1 μM). At the end of the recording, to confirm the GABAergic nature of the IPSPs, we superfused 30 μM bicuculline or 50 μM picrotoxin. These $GABA_A$ receptor antagonists completely blocked the IPSPs. To determine the stimulus–response parameters for each cell, we performed an I–O protocol (at least three times for each experimental condition at baseline, during drug administration, and during washout). We applied a range of manually adjusted currents (50–250 μA; 0.125 Hz), starting at the current required to elicit a threshold IPSP up to that required to elicit the maximal amplitude. We also applied hyperpolarizing and depolarizing voltage steps (200-pA increments, 750-msec duration) to generate voltage-current curves. Data were acquired with an Axoclamp-2A preamplifier and quantified by using the software package Clampfit 8.2 (Molecular Devices).

We examined PPF in each neuron by using 50-, 100-, and 200-msec ISIs (41). The stimulus strength was adjusted such that the amplitude of the first IPSP was 50% of maximum, as determined from the I–O relationship. We calculated the PPF ratio as the second IPSP amplitude divided by the amplitude of the first IPSP. All measures were taken before CRF or ethanol superfusion (control), during their superfusion (5–10 min), and after washout (20–30 min).

In experiments with the PKCε peptide inhibitor Tat-εV1–2 (34), we superfused 500 nM of the peptide onto CeA slices for ≈30 min before analysis of CRF and ethanol effects on IPSPs in the continued presence of Tat-εV1–2.

Whole-Cell Patch-Clamp Recording of Miniature IPSCs. In a separate set of neurons, we recorded from the CeA by using the "blind" whole-cell patch-clamp method in the presence of 10 μM CNQX, 30 μM APV, 1 μM CGP 55845A, and 1 μM tetrodotoxin (TTX) to isolate spontaneous, action potential-independent, GABAergic mIPSCs. All $GABA_A$ mIPSC recordings were made by using pipettes (input resistance 2–3 MΩ) filled with an internal solution containing: 135 mM KCl, 10 mM Hepes, 2 mM $MgCl_2$, 0.5 mM EGTA, 5 mM ATP, and 1 mM GTP (the latter two added fresh on the day of recording) (pH 7.2–7.3, osmolarity 275–290 mOsm). Data were acquired with an Axoclamp-2A preamplifier (Molecular Devices) and were analyzed by using Mini 5.1 software (Synaptosoft).

Statistical Analyses. All results are expressed as mean ± SEM values. Evoked IPSP data were analyzed by using Student's one sample or unpaired *t* tests or repeated measures ANOVA and Newman–Keuls post hoc tests, with $P < 0.05$ considered statistically significant. We evaluated results for miniature IPSCs by using cumulative probability analysis, and statistical significance was determined by using the Kolmogorov–Smirnov, nonparametric, two-sample test (42), with $P < 0.05$ considered significant.

Drugs. CGP 55845A was a gift from Novartis Pharma. We purchased D-AP5, CNQX, picrotoxin, and bicuculline from Sigma–Aldrich, TTX from Calbiochem, r/hCRF from American Peptide, Tat-εV1–2 from SynPep, and ethanol from Remet.

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