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Ethanol Alters Opioid Regulation of Ca²⁺ Influx through L-type Ca²⁺ Channels in PC12 Cells

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Abstract

Background—Studies at the behavioral and synaptic level show that effects of ethanol on the central nervous system can involve the opioid signaling system. These interactions may alter the function of a common downstream target. In this study, we examined Ca²⁺ channel function as a potential downstream target of interactions between ethanol and mu or kappa opioid receptor signaling.

Methods—The studies were carried out in a model system, undifferentiated PC12 cells transfected with mu or kappa opioid receptors. The PC12 cells express L-type Ca²⁺ channels, which were activated by K⁺ depolarization. Ca²⁺ imaging was used to measure relative Ca²⁺ flux during K⁺ depolarization and the modulation of Ca²⁺ flux by opioids and ethanol.

Results—Ethanol, mu receptor activation and kappa receptor activation all reduced the amplitude of the Ca²⁺ signal produced by K⁺ depolarization. Pretreatment with ethanol or combined treatment with ethanol and mu or kappa receptor agonists caused a reduction in the amplitude of the Ca²⁺ signal that was comparable to or smaller than that observed for the individual drugs alone, indicating an interaction by the drugs at a downstream target (or targets) that limited the modulation of Ca²⁺ flux through L-type Ca²⁺ channels.

Conclusions—These studies provide evidence for a cellular mechanism that could play an important role in ethanol regulation of synaptic transmission and behavior through interactions with the opioid signaling.

Keywords

G-protein; Ca²⁺ imaging; mu opioid receptor; kappa opioid receptor

Studies show that ethanol exerts a variety of physiological actions on the central nervous system (CNS). Many of these actions involve neurotransmitter or neuromodulator systems. One such system is the brain opioid signaling system, which functions through the interaction of the opioid peptides, beta-endorphin, enkephalins and dynorphins, with their respective cognate opioid receptors, mu (MOR), delta (DOR) and kappa (KOR). Interactions between ethanol and the opioid system have been well documented at the behavioral and synaptic level (Gianoulakis, 2009; Oswald and Wand, 2004). Ethanol exposure alters the functional properties of opioid systems through a variety of mechanisms including alterations in the biosynthesis, release, and degradation of opioid peptides, and changes in the level of opioid receptors (Gianoulakis, 2009; Mendez and Morales-Mulia, 2008). For example, acute ethanol has been reported to induce release of endogenous opioid peptides in

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brain regions associated with reward and reinforcement. The released opioid peptides are thought to play an important role in the reinforcing effects of ethanol (Koob et al., 1998; O'Brien, 2008).

At the cellular level, transmitter receptors and ion channels constitute a primary target of ethanol and opioid peptides. Interestingly, ethanol and opioid peptides often regulate the same transmitter receptor or ion channel. For example, both opioid peptides acting through opioid receptors and ethanol regulate the function of voltage-gated Ca^{2+} channels (VGCCs) (Albillos et al., 1996; Ikeda et al., 2002; Moises et al., 1994; Mullikin-Kilpatrick et al., 1995; Wang et al., 1994), inwardly rectifying K^{+} channels (GIRK channels) (Ikeda et al., 2002; Kobayashi et al., 1999; Lewohl et al., 1999; Marker et al., 2005), GABA-A receptor-mediated currents (Kumar et al., 2009; Lee et al., 2003; Wang and Randic, 1994; Xi and Akasu, 1997) and glycine receptor-mediated currents (Aguayo and Pancetti, 1994; Eggers and Berger, 2004; Guzman et al., 2009; Jeong et al., 2001; Min et al., 1996; Valenzuela et al., 1998; Ye et al., 2001). This commonality of target sites for ethanol and opioid peptides raises the possibility that actions of ethanol on the opioid signaling system could involve interactions at the level of the transmitter receptors, ion channels or signaling pathways that regulate their function. Few studies have addressed this possibility. Therefore, in the current study we have investigated potential interactions between ethanol and opioid signaling that influence the function of L-type VGCCs. For these studies we use a simplified model system, PC12 cells, a well-characterized cell line derived from a rat pheochromocytoma (Greene and Tischler, 1976; Shafer and Atchison, 1991).

Under normal culture conditions, undifferentiated PC12 cells exhibit properties of immature adrenal chromaffin cells, including limited expression of ion channels and transmitter receptors (Fujita et al., 1989). PC12 cells can be differentiated into more neuronal-like cells by treatment with growth promoting substances such as nerve growth factor (NGF) (Fujita et al., 1989). However, the undifferentiated PC12 cells provide a model with limited targets of opioid receptors, facilitating analyses. The undifferentiated PC12 cells do not normally express opioid receptors but will stably express functional receptors when subjected to standard transfection protocols (Vukojević et al., 2008a; Zarnegar et al., 2006). Previous studies have shown that in the PC12 expression system, transfected MOR can link to downstream pathways known to be involved MOR signaling (Zarnegar et al., 2006).

Our studies using Ca^{2+} imaging to measure Ca^{2+} flux through L-type VGCCs, show that in this simplified model system, ethanol, MOR and KOR all link to pathways that reduces Ca^{2+} influx through L-type VGCCs. Importantly, our studies also show that interactions occur between ethanol and MOR or KOR signaling that limit the extent of the regulation of Ca^{2+} flux. Taken together, our studies provide evidence for a cellular mechanism involving interactions between ethanol and the opioid signaling that could play an important role in the regulation of synaptic transmission and behavior when opioid systems are activated in the presence of ethanol.

METHODS AND MATERIALS

Cell culture

PC12 cells were obtained from ATCC and stably transfected with opioid receptor variants fused with enhanced green fluorescent protein (EGFP), rat MOR-EGFP (rMOR), human MOR-EGFP or rat KOR-EGFP (rKOR), as described previously (Vukojević et al., 2008a; Vukojević et al., 2008b). The cells were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum, 10% heat-inactivated horse serum, 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin (all from Invitrogen, Carlsbad, CA). For multiplication purposes, the cells were cultured in uncoated flasks (Falcon Plastics-Becton Dickinson Labware, Franklin

lakes, NJ). For Ca^{2+} imaging, the cells were plated on glass bottom culture dishes coated with poly-lysine-D (MatTek Corp, Ashland, MA, USA) and rat tail collagen (Sigma-Aldrich, San Diego, CA). The cells were maintained at 37°C in a humidified 5% CO_2 incubator. The medium was replaced every 2 to 3 days. The cells were used for studies 2 to 5 days after plating in the glass bottom culture dishes.

Ca^{2+} imaging

Intracellular Ca^{2+} levels were determined using standard microscopic fura-2 digital Ca^{2+} imaging techniques (Grynkiewicz et al., 1985) as described previously (Netzeband and Gruol, 2008). At 2–5 days in vitro, PC12 cultures were incubated for 30 min with the membrane-permeable Ca^{2+} -sensitive dye fura-2 AM (Molecular Probes-Invitrogen, Carlsbad, CA) at $3\text{ }\mu\text{M}$ in physiological saline containing 0.02 % pluronic F-127 (Molecular Probes) at room temperature in the dark. The composition of the physiological saline was: 140 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4 , 1.25 mM Na_2HPO_4 , 2.2 mM CaCl_2 , 2 mM MgSO_4 , 10 mM glucose and 10 mM HEPES. Prior to starting the experiments, the cultures were incubated in dye-free saline for at least 45 minutes after removal of fura-2 in order to allow for the de-esterification of fura-2AM.

For Ca^{2+} imaging experiments, the culture dish containing the cells was mounted on the stage of an inverted fluorescence microscope and viewed using a $40\times$ (1.3 NA) oil immersion objective. Fluorescent images of live PC12 cells were acquired at 510 nm, using excitation wavelengths of 340 and 380 nm. A SIT-66 or VE-1000 video camera (640 pixels $\times$ 480 lines; 8 bit, 256 gray levels) (DAGE-MTI, Dage, Michigan City, IN) was used. The images were digitized for real-time display using the MCID imaging software (Imaging Research, Inc., St-Catharines, Ontario, Canada). Images were acquired at 0.8 s intervals (4 frames per wavelength were averaged for each time point). Intracellular Ca^{2+} levels in individual PC12 cells were calculated by converting the fluorescence intensity ratios (340/380 nm) to intracellular Ca^{2+} concentrations. The following formula was used for this conversion: $[\text{Ca}^{2+}]_i = K_d(R - R_{\min}) / (R_{\max} - R) * F_o / F_s$, where R is the ratio value, $R_{\min}$ is the ratio for a Ca^{2+} free solution, $R_{\max}$ is the ratio for a saturated Ca^{2+} solution, K_d is 225 (the dissociation constant for fura-2), F_o is the intensity of a Ca^{2+} free solution at 380 nm and F_s is the intensity of a saturated Ca^{2+} solution at 380 nm. Calibration was performed using fura-2 salt solutions with known Ca^{2+} concentrations (Molecular Probes-Invitrogen calibration kit). Typical $R_{\max}$, $R_{\min}$, and F_o/F_s values were 0.40, 1.19 and 1.82, respectively. The low level of background fluorescence and adjustment of the black level of the SIT camera eliminated the need for background subtraction methods. All experiments were performed at room temperature. Unless otherwise noted, all chemicals were of reagent grade and purchased from Sigma-Aldrich.

Intracellular Ca^{2+} levels were measured in the PC12 cells under baseline (control) resting conditions and after stimulation of the cells with high K^+ saline to depolarize the cells. The membrane depolarization activated VGCCs resulting in Ca^{2+} influx through the VGCCs. The K^+ saline (50–100 mM) was prepared by substitution of KCl for NaCl in physiological saline. K^+ was applied by a brief micropressure pulse (Picospritzer II; Parker Hannifin Corporation, Fairfield, NJ) from a glass micropipette (1–2 μm tip) positioned to expose the target cell to the K^+ application. Fast green (0.03%; Sigma) was included in the K^+ saline to monitor exposure. Control experiments showed that when fast green in vehicle (saline) was tested by micropressure application, there was no effect on the measured fluorescence or excitability of the cells.

Before experiments were started, K^+ application and the superfusion rate for the bath saline were adjusted to optimize the Ca^{2+} recording and were not changed during the experiment. The superfusion rate was approximately 1.5 ml/min and was maintained throughout the

experiment. The K^+ pipette was placed in a standardized position relative to the cell to be studied and the duration of K^+ application was adjusted, typically between 0.7 and 1.1 s. By increasing the duration of K^+ application, the concentration of K^+ that the cell was exposed to increased, thereby increasing the membrane depolarization up to a maximum (Fig. 1B). The duration of the K^+ application was set to evoke a Ca^{2+} signal in the recorded cell that was between 200 to 800 nM, submaximal levels for Ca^{2+} influx in the PC12 cells and a level that can be detected by our Ca^{2+} imaging system without reaching saturation. Experiments were carried out on cells that showed stable Ca^{2+} signals to K^+ depolarization during control conditions. During experiments, K^+ stimulation was applied continuously to the cells at a rate of once every three minutes. For each K^+ stimulus, measurement of intracellular Ca^{2+} levels started approximately 8 s before K^+ application and continued for 30–40 s, at which time intracellular Ca^{2+} was approaching baseline levels. For each K^+ stimulus, measurements were made of resting Ca^{2+} level and the peak amplitude of the Ca^{2+} signal (rest subtracted), time to peak amplitude and duration at half maximal peak amplitude. Drug effects were considered to be stable when the magnitude of the Ca^{2+} signal was similar for two stimulations (6 min) or more.

Electrophysiological recording

Perforated-patch whole-cell current-clamp recordings were made from the PC12 cells using standard techniques as described previously (Netzeband and Gruol, 2008). The patch pipette solution contained: NaCl 6 mM, K^+ -methylsulfate 140 mM, $MgCl_2$ 2 mM, $CaCl_2$ 0.5 mM, HEPES-KOH 10 mM and glucose 10 mM. Tips of patch pipettes (4–5 M Ω) were filled first with antibiotic-free solution and the pipettes were then back-filled with the same solution only containing the pore-forming antibiotics nystatin or amphotericin B (200 μ g/ml; Calbiochem, San Diego, CA). The bath saline was the same physiological saline as used for Ca^{2+} imaging studies. In some studies, the electrophysiological recordings were carried out simultaneously with Ca^{2+} imaging experiments. The cells were loaded with fura-2 prior to initiating the current clamp recordings. Recordings were performed using the Axopatch-1C amplifier and digitized (1 kHz) using the Digidata 1322A interface and pCLAMP v.8.0 software (all from Axon Instruments-Molecular Devices, Sunnyvale, CA). All recordings were made at room temperature (23–25°C).

Drugs

A variety of drugs were tested by superfusion including DAMGO (Tocris Cookson, Ltd., Ellisville, MO), dynorphin A (Dyn A), ethanol (Remet Alcohols, La Mirada, CA), the L-type Ca^{2+} VGCC blocker nimodipine (Research Biochemicals International, Natick, MA) and ryanodine (Tocris). Ryanodine was dissolved in DMSO at a 1000X stock concentration and diluted 1:1000 in bath saline for use. For vehicle control, DMSO was diluted 1:1000 in bath saline for use. For experiments with receptor ligands and ethanol, the cells were exposed to the ligand and ethanol only once (i.e., repeated application of the same drug was not used).

Statistical analysis

Statistical significance ($p \leq 0.05$) was determined using the one-group Student's t-test (normalized data; control = 1), paired Student's t-test or ANOVA. Data are reported as means $\pm$ standard error of the mean (SEM). n = number of cells measured. All studies involved at least 2 to 7 different cultures.

RESULTS

The functional properties of opioid receptors and interactions with ethanol were measured in rMOR, hMOR and rKOR transformed PC12 cells by testing the effect of the selective

receptor agonists and ethanol on Ca^{2+} influx through VGCCs. Ca^{2+} influx was measured by Ca^{2+} imaging, a technique that does not produce prominent effects on the intracellular compartment and signal transduction pathways that could be involved in the actions of opioid receptor ligands or ethanol. The level of expression of opioid receptors in the transformed cells varied across cells (Fig. 1A,4A,6A), as did the magnitude of Ca^{2+} signal produced by activation of VGCCs, as noted previously (Grant et al., 1993), and the effects of receptor ligands. To adjust for this variability, measurements made during drug superfusion were normalized to values measured during the control period of saline superfusion. Normalized data from similar experiments were combined.

rMOR functionality in PC-12 cells

To examine the functional properties of rMOR in the transformed PC12 cells, the effect of the selective MOR agonist DAMGO on Ca^{2+} influx through VGCCs was studied. Expression of rMOR in the PC12 cells was confirmed by fluorescence imaging for EGFP (Fig. 1A). Activation of VGCCs was achieved by brief (sec) micropressure application of physiological saline containing a high concentration of K^+ (100 mM; Na^+ levels reduced) to depolarize the cell membrane. Whole cell patch clamp recordings (perforated patch) from the PC12 cells confirmed that brief application of high K^+ saline produced a transient membrane depolarization. The depolarization varied in amplitude depending on the duration of K^+ application (Fig. 1B). Combined Ca^{2+} imaging and electrophysiological recordings showed that a Ca^{2+} signal was associated with membrane depolarization produced by K^+ , consistent with the activation of VGCCs by the K^+ depolarization (Fig. 1C).

The primary VGCC expressed by undifferentiated PC12 cells is the L-type VGCC (Mullikin-Kilpatrick and Treistman, 1995). The Ca^{2+} signal evoked by K^+ depolarization was significantly reduced (~90%) by the selective L-type VGCC blocker nimodipine (1–2 μM ; Fig. 1D), consistent with a prominent role for L-type VGCCs in the generation of the Ca^{2+} signal. Addition of DAMGO (2 μM) to the bath saline did not alter the amplitude or duration of the membrane depolarization produced by K^+ (Fig. 1E, F). Resting membrane potential (Fig. 1G) and input resistance were also unaltered by DAMGO (Fig. 1H–J), indicating that DAMGO did not affect passive membrane properties of the PC12 cells.

The effect of DAMGO on Ca^{2+} influx through L-type VGCCs in the PC12 cells was determined by comparing the magnitude of the Ca^{2+} signals produced by K^+ depolarization during control saline superfusion with the magnitude during superfusion with DAMGO (2 or 5 μM). Measurements were made of the peak amplitude (resting levels subtracted), time to peak and duration (measured at half maximum peak amplitude) of the Ca^{2+} signals. The peak amplitude of the Ca^{2+} signal evoked by K^+ was reduced (~15–30%) transiently by DAMGO in a dose-dependent manner (Fig. 2A–D). The reduction by DAMGO had a slow onset (in part due to the time needed to exchange the bath, ~3 min) and reached a maximum within approximately 10 min (Fig. 2A). The effect on the peak amplitude declined in spite of the continuous presence of DAMGO, presumably due to internalization of the activated receptor or desensitization of downstream signal transduction (Fig. 2A). Time to peak and duration of the Ca^{2+} signal were unaltered by DAMGO (Fig. 2E). Resting Ca^{2+} levels were not significantly affected by DAMGO (Fig. 2F).

The reduction of the peak amplitude of the Ca^{2+} signal produced by DAMGO was not observed in cells pretreated with the MOR antagonist naloxone (2 μM), consistent with the involvement of MOR in the action of DAMGO (Fig. 2G). Time to peak and duration of the Ca^{2+} signals were similar for cells treated with naloxone and naloxone plus DAMGO (Fig. 2H). Mean resting Ca^{2+} levels were 99 ± 5 nM ($n=28$) and 110 ± 7 nM ($n=28$) in the presence of 2 μM naloxone and 2 μM naloxone plus 2 μM DAMGO, respectively.

Ethanol-MOR interaction

Previous voltage clamp studies showed that ethanol exposure reduces Ca^{2+} currents through L-type VGCCs in undifferentiated PC-12 cells (Mullikin-Kilpatrick and Treisman, 1995; Wang et al., 1994). Consistent with these results, superfusion of ethanol reduced the peak amplitude of the Ca^{2+} signal evoked by K^+ in the rMOR transfected PC12 cells (Fig. 3A, B, E, F). The reduction in the peak amplitude had a slow onset and reached a maximum effect at ~10 min, after which it declined although ethanol was still present (Fig. 3A, F). These results indicate that MOR and ethanol have a similar downstream target, L-type VGCCs, a situation that could result in an additive reduction in Ca^{2+} influx through L-type VGCCs if MOR was activated under conditions of ethanol exposure. To determine if such additive actions between ethanol and MOR occur, the magnitude of the Ca^{2+} signal evoked by K^+ was measured under conditions of ethanol superfusion alone or superfusion of ethanol plus DAMGO. In the presence of ethanol, DAMGO reduced (~10–15%) the peak amplitude of the Ca^{2+} signal but the reduction in the presence of DAMGO and ethanol was similar to that observed for ethanol alone (Fig. 3A, B, E, F). Thus, there was no additive effect of DAMGO and ethanol. These results indicate that interactions occur when MOR is activated in the presence of ethanol that limit the regulation of L-type Ca^{2+} channel function in PC12 cells. Mean time to peak and duration of the Ca^{2+} signals were unaltered by these treatments (Fig. 3C, G). Resting Ca^{2+} levels were increased by exposure to ethanol and ethanol plus DAMGO (Fig. 3D, H).

Intracellular stores and the actions of ethanol and DAMGO

Influx of Ca^{2+} through VGCCs can activate ryanodine receptors (RyR) on the endoplasmic reticulum resulting in Ca^{2+} release from intracellular stores. Therefore, Ca^{2+} derived from intracellular stores could contribute to the Ca^{2+} signal produced by K^+ depolarization and could be a site of DAMGO and ethanol action. Ethanol has been reported to increase Ca^{2+} release from intracellular stores in PC12 cells (Belia et al., 1995; Rabe and Weight, 1988), neurons (Kelm et al., 2007; Mironov and Hermann, 1996) and astrocytes. (Salazar et al., 2008). Such an effect could impact interpretation of the actions of ethanol on Ca^{2+} signals evoked by K^+ depolarization. To determine if effects of ethanol on Ca^{2+} release from stores contributed to our results, we carried out studies using the RyR blocker ryanodine. These studies were carried out in PC12 cells transfected with hMOR. The hMOR cells expressed a greater number of L-type VGCCs than the rMOR transfected PC12 cells, as evidenced by the large (>1 μM) Ca^{2+} signal evoked by K^+ depolarization with 100 mM K^+ saline, which was used in the studies of rMOR transfected cells. For experiments with the hMOR transfected cells, the concentration of K^+ used in the high K^+ saline was reduced to 60 mM, a concentration that produced a Ca^{2+} signal between 200 and 800 nM, comparable to that the Ca^{2+} signals evoked by 100 mM K^+ in the rMOR cells.

Application of K^+ by micropressure produced a Ca^{2+} signal in the hMOR cells similar to that observed in the rMOR cells (compare Fig 2B, C with Fig. 4B, C). The Ca^{2+} signal was almost completely blocked (~90%) by 2 μM nimodipine, consistent with L-type VGCCs as the primary VGCC mediating the Ca^{2+} signal (Fig 4D). As was observed in the rMOR PC12 cells (Fig. 1D), superfusion of DAMGO (1 μM) reduced (~20%) the peak amplitude of the K^+ evoked Ca^{2+} signal (Fig. 4D), with no effect on time to peak or duration of the Ca^{2+} signal (Fig. 4E) or resting Ca^{2+} levels (Fig. 4F). The effect of DAMGO was typically larger and more prolonged than the effect observed in rMOR PC12 cells. These differences are likely to be due to greater expression of hMOR, L-type VGCCs and biochemical pathways involved in MOR regulation of L-type VGCCs in the hMOR PC12 cells compared with the rMOR PC12 cells.

Superfusion with ryanodine (25 μ M) reduced (~10 to 20%) the amplitude of the K^+ -evoked Ca^{2+} signal, indicating that Ca^{2+} release from intracellular stores contributed to the Ca^{2+} signal (Fig 5A). However, in the presence of ryanodine, the effect of DAMGO on the K^+ -evoked Ca^{2+} signal (~20% reduction) was comparable to that observed in the absence of ryanodine (compare Fig. 5A2 with Fig. 4D). Ethanol was also effective in reducing the Ca^{2+} signal produced by K^+ depolarization in the presence of ryanodine (Fig. 5B), reducing the peak amplitude of the Ca^{2+} signal ~20 %, similar to the effect of DAMGO. When DAMGO and ethanol were applied in the presence of ryanodine, a reduction of the K^+ evoked Ca^{2+} signal was still observed (Fig. 5C). However, the magnitude of the reduction (~20%) was similar to that produced by DAMGO or ethanol alone and did not show an additive effect (Fig. 5C2), as was observed in the rMOR experiments. These results indicate that Ca^{2+} release from stores is not a prominent factor in the interactions that occur between ethanol and MOR signaling that limit the effect of these drugs on L-type Ca^{2+} channels in PC12 cells.

Mean time to peak and duration of the Ca^{2+} signals were not significantly altered by these treatments (Fig. 5A3, B3, C3). Resting Ca^{2+} levels were significantly increased (ANOVA, $p < 0.05$) by exposure to ryanodine and ethanol (~13%, ryanodine vs. control, $n = 21$; ~17% ethanol plus ryanodine vs. ryanodine, $n = 13$; ~17%, ryanodine plus ethanol plus DAMGO vs. ryanodine, $n = 21$). This effect of ryanodine on resting Ca^{2+} levels is likely due to the ability of ryanodine to transiently activate RyRs causing release of intracellular Ca^{2+} from stores prior to its action as blocker of the open RyR channel. (Kano et al., 1995). Resting Ca^{2+} levels were not significantly increased by DAMGO in the presence of ryanodine ($n = 19$).

Dynorphin A reduces K^+ evoked Ca^{2+} signal

In addition to MOR, studies show that KOR can also modulate Ca^{2+} flux through VGCCs (Kitamura et al., 2002; Tallent et al., 1994; Velazquez-Marrero et al.). Therefore, it was of interest to determine if ethanol could also interacted with KOR regulation of Ca^{2+} influx through L-type Ca^{2+} channels in transformed PC12 cells. For these studies, undifferentiated PC12 cells stably expressing rKOR-EGFP were used. Expression of KOR in cells selected for study was confirmed by fluorescence imaging for EGFP (Fig. 6A). As for the MOR transformed PC12 cells, in the rKOR transformed PC12 cells K^+ depolarization elicited a transient Ca^{2+} signal (Fig. 6B). Like the hMOR PC12 cells, the KOR cells also expressed more L-type VGCCs than the rMOR, as evidenced by a large response to 100 mM high K^+ saline. Therefore, the concentration of K^+ in the high K^+ saline was reduced to 50 mM, a concentration that produced a Ca^{2+} signal similar in magnitude to that observed in the hMOR cells. The L-type Ca^{2+} channel blocker nimodipine significantly reduced the amplitude of the K^+ -evoked Ca^{2+} signal in the rKOR (~90%) transformed cells (Fig. 6D), demonstrating a prominent role for L-type VGCCs in the generation of the Ca^{2+} signal.

The selective KOR agonist Dyn A (200 nM) applied by superfusion significantly reduced (~25%) the peak amplitude of the K^+ -evoked Ca^{2+} signal (Fig. 6B, C, E). Time to peak and duration of the Ca^{2+} signals were similar for cells treated with saline or Dyn A (Fig. 6F). Resting Ca^{2+} levels were unaltered by Dyn A (Fig. 6G).

Ethanol-KOR interaction

Superfusion with 20 mM ethanol also significantly reduced (~20%) the peak amplitude of the Ca^{2+} signal evoked by K^+ in the KOR transformed cells (Fig. 7A–C). The effect of ethanol was similar to that observed for Dyn A (compare Figs. 6 & 7). Mean values for time to peak and duration were not altered by ethanol (time to peak = 3.6 ± 0.1 s for both control and ethanol; $n = 23$ for each condition; duration = 7.2 ± 0.4 s for both control and ethanol; $n = 23$ for each condition). Resting Ca^{2+} levels were significantly increased by ethanol

($p < 0.05$, paired t-test). Mean values were 49 ± 3 nM and 58 ± 3 nM ($n = 22$ under each condition) for control and ethanol conditions, respectively.

To determine if an interaction occurred between ethanol and KOR signaling that affected the regulation of Ca^{2+} influx through L-type VGCCs by ethanol (20 mM) or Dyn A, both drugs were applied simultaneously by superfusion. Although Dyn A plus ethanol reduced (~20%) the peak amplitude of the Ca^{2+} signal produced by K^+ depolarization, the effect was not a summation of the individual effects of each drug. Instead, the magnitude of the reduction of the Ca^{2+} signal was similar to that observed with either agent alone (Fig. 5), results similar to that observed for ethanol and MOR. Mean values for time to peak and duration were not altered by ethanol plus Dyn A (time to peak = 3.9 ± 0.2 s for both control and ethanol plus Dyn A, $n = 23$ for each condition; duration = 8.7 ± 0.5 s for both control and ethanol plus Dyn A, $n = 23$ for each condition). A small but significant ($p < 0.05$, paired t-test) increase in resting Ca^{2+} levels was produced by Dyn A plus ethanol (control = 67 ± 6 nM, $n = 23$; Dyn A plus ethanol = 73 ± 6 nM, $n = 23$).

DISCUSSION

Interactions between ethanol and opioids are well documented at the behavioral level (Gianoulakis, 2009; Oswald and Wand, 2004; Wee and Koob). However, few studies have determined if such interactions occur at the level of opioid receptor signaling. In the current study, we used PC12 cells transformed to express MOR or KOR as a simplified model system to address this issue. Our studies show that ethanol, MOR and KOR are all linked to a downstream pathway that regulates L-type VGCCs in the transformed PC12 cells. Thus, activation of MOR or KOR with selective receptor agonists reduced the Ca^{2+} signal produced by K^+ depolarization, an experimental manipulation that activates VGCCs resulting in Ca^{2+} influx. Ethanol also reduced the Ca^{2+} signal produced by K^+ depolarization in the transformed PC12 cells. These effects varied in magnitude for the three transformed cell lines studied (rMOR, hMOR and rKOR), presumably due to differences in the level of expression of opioid receptor, Ca^{2+} channels or signal transduction molecules that mediate the effects of ethanol and opioid receptor activation. The magnitude of the Ca^{2+} signal reduction was similar for ethanol and hMOR or KOR activation in the respective transformed PC12 cells. Importantly, when MOR or KOR was activated during exposure to ethanol, the extent of the reduction of Ca^{2+} signal was not a summation of the effects of MOR or KOR activation plus ethanol. Instead, the extent of the reduction was similar to or smaller than that observed for the individual drugs alone. These results show that there is an interaction between ethanol and opioid receptor signaling that limits the magnitude of the regulation of L-type VGCCs function by these drugs.

Both opioids and ethanol are known to regulate the function of Ca^{2+} channels (Albillos et al., 1996; Bourinet et al., 1996; Littleton et al., 1992; Mullikin-Kilpatrick and Treistman, 1995; Piros et al., 1996; Rola et al., 2008; Twombly et al., 1990; Walter and Messing, 1999). Other ion channels such as GIRK channels are also a common downstream target of opioids and ethanol (Aryal et al., 2009; Kobayashi et al., 1999; Lewohl et al., 1999). Undifferentiated PC12 cells have been shown to express L-type Ca^{2+} channels (Mullikin-Kilpatrick and Treistman, 1995) and K^+ channels but few Na^+ channels (Garber et al., 1989; Hoshi and Aldrich, 1988; Strubing and Hescheler, 1996). In our studies, the expression of L-type Ca^{2+} channels in the transformed cells was revealed by the association of Ca^{2+} influx with the membrane depolarization produced by high K^+ saline and the blockade of the Ca^{2+} influx by nimodipine, an antagonist of L-type VGCCs. Activation of MOR by DAMGO reduced the Ca^{2+} influx but did not alter resting membrane potential, input resistance or the characteristics of the K^+ evoked membrane depolarization. These results suggest that the

primary downstream target of MOR activation in the undifferentiated PC12 cells was L-type VGCCs.

Ca²⁺ imaging studies showed that ethanol and Dyn A activation of KOR also reduced the Ca²⁺ signal produced by K⁺ depolarization in the KOR transformed cells. Although current clamp recordings of membrane properties and K⁺ depolarization were not carried out in the studies of ethanol and Dyn A, any changes in membrane properties produced by ethanol or Dyn A are likely to be small. Neither ethanol nor Dyn A altered the time to peak or duration of the K⁺ evoked Ca²⁺ signal, parameters that would be altered by prominent changes in membrane properties. Ethanol increased resting Ca²⁺ levels in the KOR cells, an effect that could result from membrane depolarization, but this effect was small (~10 nM increase) and likely to reflect actions of ethanol on Ca²⁺ release from intracellular stores (Belia et al., 1995; Rabe and Weight, 1988). Therefore, it is unlikely that changes in membrane properties were a major contributor to the regulation of Ca²⁺ influx through VGCCs by ethanol.

The action of both ethanol and DAMGO on the K⁺ evoked Ca²⁺ signal showed a relatively long onset (several minutes), consistent with the involvement of a second messenger pathway. When ethanol and DAMGO were applied together, an additive effect was expected. However, this was not observed. Instead, the reduction produced by DAMGO plus ethanol was similar to or smaller than that produced by DAMGO or ethanol alone. Comparable results were obtained when ethanol was applied simultaneously with DAMGO. In the case of Dyn A, ethanol and Dyn A were applied simultaneously. Again, there was no additive effect of ethanol and Dyn A on the K⁺ evoked Ca²⁺ signal. These results show that there is an interaction between ethanol and MOR or KOR signaling that limits the modulation of Ca²⁺ flux by opioids and/or ethanol. The site of this interaction and mechanism through which the interaction occurs remains to be determined.

Both DAMGO and Dyn A are known to produce their downstream effects by activating a Gi/Go protein, which induces dissociation of the G-protein into Gα-GTP and Gβγ subunits. Both Gα-GTP and Gβγ subunits have downstream actions. The Gβγ subunit can regulate the activity of VGCCs and other ion channels through a direct membrane delimited pathway or through activation of enzymes such as PKC. The Gα subunit also regulates second messenger pathways, and in particular, inhibition of adenylate cyclase (Baldelli et al., 2004; Jiang and Bajpayee, 2009; Luttrell, 2008). Receptor signaling is transient due to regulatory processes that terminate the signaling. These processes include phosphorylation, desensitization, endocytosis, and downregulation (Kelly et al., 2008).

Previous studies have provided evidence for the involvement of Gi proteins in the regulation of L-type Ca²⁺ channels and effects of ethanol in undifferentiated PC12 cells (Mullikin-Kilpatrick et al., 1995). For example, intracellular administration of the non-hydrolyzable analogue GTPγS, a compound that activates G proteins, reduced the Ca²⁺ currents recorded in voltage clamped PC12 cells (Mullikin-Kilpatrick et al., 1995), consistent with the ability of G-proteins to regulate Ca²⁺ currents in PC12 cells. Gi/Go proteins were also shown to directly regulate L-type Ca²⁺ channels through a membrane delimited pathway in adrenal chromaffin cells (Baldelli et al., 2004; Carbone et al., 2001), the parent cell type from which PC12 cells were derived. Pretreatment of PC12 cells with pertussis toxin (PTX), a compound that blocks Gi/Go protein activation, or intracellular administration of guanosine-5'-O-(2-thio)diphosphate (GDPβS), a compound that blocks activation of G proteins, reduced ethanol inhibition of L-type Ca²⁺ currents recorded in voltage clamped PC12 cells (Mullikin-Kilpatrick et al., 1995), showing that G-protein activation is an important aspect of ethanol regulation of Ca²⁺ currents. Moreover, studies with Giα specific antibodies, identified Giα₁ and Giα₂ as the specific Giα proteins involved in ethanol

inhibition of L-type Ca^{2+} channels in undifferentiated PC12 cells (Mullikin-Kilpatrick et al., 1995). Previous studies in other systems have also provided evidence for a role for G-proteins in ethanol regulation of VGCC function. Thus, in dorsal root ganglia neurons obtained from embryonic mice intracellular dialysis with polyclonal antibodies against the α -subunit of G_o , but not G_s , abolished the inhibitory effect of ethanol on Ca^{2+} currents (Huang and McArdle, 1994). Similarly, in *Helix* neurons acute ethanol reduced Ca^{2+} currents through a transduction pathway involving G-protein, cytoplasmic Ca^{2+} , and protein kinase C (Kerschbaum and Hermann, 1997). Dopamine also suppressed Ca^{2+} currents in the *Helix* neurons and this effect involved G_o . Dopamine suppression of Ca^{2+} currents prevented the reduction of the Ca^{2+} currents by ethanol, indicating a common transduction pathway for ethanol and dopamine via G_o -protein in *Helix* neurons (Kerschbaum and Hermann, 1997). Ethanol has also been reported to activate G-proteins (Hatta et al., 1994; Hoek and Rubin, 1990; Hoffman and Tabakoff, 1990; Klinker et al., 1996; Lahnsteiner and Hermann, 1995).

Recently we showed that acute ethanol alters the status of agonist-free rMOR in the membrane of transformed PC12 cells. Acute ethanol transiently increased the mobility of rMOR and its surface density, an effect that lasted 20–30 min, after which receptor density decreased to lower than pre-ethanol levels, presumably due to receptor internalization (Vukojević et al., 2008a; Vukojević et al., 2008b). Acute ethanol also altered lipid dynamics of the membrane in rMOR transformed PC12 cells, causing an effect on lipid dynamics that was similar to the effect of methyl- β -cyclodextrin (M β CD), a compound that extracts cholesterol and disrupts lipid raft domains of the membrane (Vukojević et al., 2008a; Vukojević et al., 2008b). Studies have shown that MOR is located the lipid raft domain of the plasma membrane where it is tethered to $\text{Gi}\alpha_2$ (Ge et al., 2009). A lipid raft location of MOR was shown to be critical for G-protein coupled signaling (Zhao et al., 2006; Zheng et al., 2008). Treatment with M β CD translocates $\text{Gi}\alpha$ and MOR to non-raft membrane domains (Zheng et al., 2008). L-type Ca^{2+} channels have also been shown to be localized in lipid raft domains (Park and Kim, 2009) where they are tethered to $\text{Gi}\alpha_2$ (Ge et al., 2009). Thus, ethanol actions on G-proteins or lipid rafts could play an important role in the interaction between MOR signaling and ethanol observed in our study. Interestingly, the MOR antagonist naloxone blocks the effects of ethanol on plasma membrane lipid dynamics in the MOR transformed PC12 cells and stabilizes MOR in the membrane domains (Vukojević et al., 2008b). Thus, naloxone may stabilize the MOR-G protein complex thereby blocking the ability of ethanol to interact with Gi . Future studies will be required to test this possibility and to determine the mechanisms mediating the ethanol-opioid receptor interactions observed in the current studies.

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Figure 1.

Effect of DAMGO on membrane depolarization produced by high K^+ saline (100 mM) in rMOR transformed PC12 cells. A. Digitized images of live PC12 cells used for electrophysiological studies. The fluorescence image shows expression of MOR-EGFP, which is primarily localized at the cell membrane. B. Current clamp recordings showing membrane depolarizations produced by K^+ saline applied at the arrow by brief micropressure application at different durations. Increasing the duration of application increased the depolarization. C. Simultaneous current clamp (bottom panel) and Ca^{2+} recordings (top panel) during K^+ depolarization showing that the membrane depolarization elicited a Ca^{2+} signal. D. Effect of the L-type VGCC blocker nimodipine (nimod) on the K^+ evoked Ca^{2+} signal. Graph shows mean values ($\pm$ SEM) for the peak amplitude of the K^+ evoked Ca^{2+} signal under control conditions and in the presence of nimodipine. Inset shows representative Ca^{2+} signals before and after nimodipine application. Nimodipine significantly reduced the Ca^{2+} signal indicating that the primary VGCC responsible for the Ca^{2+} signal was the L-type VGCC. Asterisk indicates significant difference from control values ($p < 0.05$, unpaired t-test). E. Mean peak amplitude ($\pm$ SEM) of the membrane depolarization produced by different durations of K^+ application before and after addition of DAMGO (1 μM) to the recording saline. Representative recordings are shown to the right. DAMGO did not significantly alter the peak amplitude of the membrane depolarization. F. Mean values ($\pm$ SEM) for the amplitude and duration of the K^+ -evoked membrane depolarization before and after application of 2 μM DAMGO. G. Mean values ($\pm$ SEM) for resting membrane potential before and after application of 2 μM DAMGO. H. Current clamp recordings showing the membrane response produced by intracellular application of depolarizing and hyperpolarizing current pulses under control conditions and in the presence

of DAMGO (1 μ M). Depolarization of the membrane did not produce spike events due the immature status of the cells (H, bottom panels). I. A representative current-voltage relationship derived from measurement of voltage responses produced by intracellular current injection as shown in H. J. Mean values ($\pm$ SEM) for membrane input resistance before and after application of 2 μ M DAMGO. Input resistance was calculated from the slope of current-voltage curves such as that shown in I. For all graphs, numbers in bars show the number of cells studied.

Figure 2.

DAMGO inhibition of K^+ evoked Ca^{2+} signal. A. Normalized mean peak amplitude ($\pm$ SEM, $n=32$) of the K^+ (100 mM) evoked Ca^{2+} signal under control conditions (saline superfusion), after superfusion of 2 or 5 μ M DAMGO and during washout with saline (wo). The two doses were tested on different populations of cells. In each experiment, the peak amplitude of the Ca^{2+} signal was normalized to the mean value for the peak amplitude during control conditions on an individual cell basis. Horizontal arrows show time of saline and DAMGO superfusion and washout. In this and other figures, the dotted line shows control levels. DAMGO reduced the peak amplitude of the Ca^{2+} signal in a dose-dependent manner. B, C. Representative Ca^{2+} signals recorded in 4 cells under control (B) conditions and in the presence of 2 μ M DAMGO (C). K^+ (2 s application) was applied at the arrow. D. Normalized mean values for peak amplitude ($\pm$ SEM) of the K^+ evoked Ca^{2+} signal under control conditions (at time = 0), in the presence of 2 μ M DAMGO and 5 μ M DAMGO. Asterisk indicates significant difference from control values; the hatched character indicates significant difference from 2 μ M DAMGO ($p < 0.05$, ANOVA). E. Mean values ($\pm$ SEM) for time to peak (TTP) and duration (measured at half maximum peak amplitude) of the Ca^{2+} signals measured under control conditions and in the presence of DAMGO. F. Mean values ($\pm$ SEM) for resting Ca^{2+} levels under control conditions and in the presence of DAMGO. G. Normalized mean peak amplitude ($\pm$ SEM, $n = 22$) of the K^+ evoked Ca^{2+} signal during superfusion of 2 μ M naloxone and after superfusion of DAMGO (2 μ M) plus naloxone. In each experiment, the peak amplitude of the Ca^{2+} signal was normalized to the mean value for the peak amplitude during control (naloxone alone) conditions on an individual cell basis. Horizontal arrows show the time of naloxone and naloxone plus DAMGO superfusion. Naloxone blocked the effect of DAMGO. H. Mean values ($\pm$ SEM) for TTP and duration (measured at half maximum peak amplitude) of the K^+ evoked Ca^{2+} signals measured in the presence of naloxone (Nal) and naloxone plus DAMGO. In graphs D, E, F and H, control values represent an average of all control measurements; drug values represent the peak effect. For all graphs, numbers in bars show the number of cells studied.

Figure 3.

Interactions between ethanol and DAMGO. A, E. Normalized mean values for the peak amplitude ($\pm$ SEM) of the K^+ (100 mM) evoked Ca^{2+} signal under control conditions (saline superfusion) and superfusion of 20 mM ethanol or 20 mM ethanol plus 2 μM (A) or 5 μM (E) DAMGO. In each experiment, the peak amplitude of the Ca^{2+} signal was normalized to the mean value for the peak amplitude during control conditions on an individual cell basis. Horizontal arrows show the time of saline, ethanol and ethanol plus DAMGO superfusion. The peak amplitude of the Ca^{2+} signal was similar in ethanol alone and ethanol plus DAMGO. B, F. Normalized mean values for peak amplitude ($\pm$ SEM) of the K^+ evoked Ca^{2+} signals under control conditions (at time = -20), in the presence of 20 mM ethanol (at time = -6 min for 2 μM DAMGO (B); at time = -8 for 5 μM DAMGO (F)) and in the presence of 20 mM ethanol plus DAMGO (at time = 10 min for 2 μM DAMGO (B); at time = 8 for 5 μM DAMGO (F)). Dotted line shows control levels. C, G. Mean values ($\pm$ SEM) for time to peak (TTP) and duration (measured at half maximum peak amplitude) of the Ca^{2+} signals measured under control conditions, in the presence of 20 mM ethanol and in the presence of 20 mM ethanol plus 2 μM DAMGO (C) or 20 mM ethanol plus 5 μM DAMGO (G). D, H. Mean values ($\pm$ SEM) for resting Ca^{2+} levels under control conditions (D, H), in the presence of 20 mM ethanol and in the presence of 20 mM ethanol plus 2 μM DAMGO (D) or 20 mM ethanol plus 5 μM DAMGO (H). For all graphs, numbers in bars show the number of cells studied. Asterisk indicates significant difference from control value ($p < 0.05$, ANOVA).

Figure 4.

DAMGO inhibition of Ca^{2+} signal produced by K^{+} saline in hMOR transformed PC12 cells. A. Digitized fluorescence image of live hMOR transformed PC12 cells used for Ca^{2+} imaging studies. The image shows expression of hMOR-EGFP, which is primarily localized at the cell membrane. B, E. Normalized mean peak amplitude values ($\pm$ SEM) of the K^{+} evoked (60 mM) Ca^{2+} signal under control conditions (saline superfusion), during superfusion of 1 μM DAMGO and during washout with saline (wo). In each experiment, the peak amplitude of the Ca^{2+} signal was normalized to the mean value for the peak amplitude during control conditions on an individual cell basis. Horizontal arrows show time of saline (cont) and DAMGO superfusion and washout. In E, a short washout (wo) period is followed by superfusion of the L-type VGCC blocker nimodipine (nimod). Nimodipine significantly reduced the Ca^{2+} signal indicating that the primary VGCC responsible for the Ca^{2+} signal was the L-type VGCC. C, D. Representative Ca^{2+} signals evoked by K^{+} saline (applied at the arrow) in hMOR PC12 cells during control conditions (saline superfusion) (C) and after superfusion of DAMGO (D). Responses of 4 cells within a microscopic field are shown. F. Graph showing mean values ($\pm$ SEM) for the peak amplitude of the K^{+} -evoked Ca^{2+} signal under control conditions and in the presence of DAMGO and nimodipine. Dotted line shows control levels. Asterisk indicates significant difference from conditions without nimodipine ($p < 0.05$, ANOVA). G. Mean values ($\pm$ SEM) for time to peak (TTP) and duration (measured at half maximum peak amplitude) of the K^{+} -evoked Ca^{2+} signal under control conditions and in the presence of DAMGO. H. Mean values ($\pm$ SEM) for resting Ca^{2+} levels under control conditions and in the presence of DAMGO. For all graphs, numbers in bars show the number of cells studied. In graphs shown in F–H, control values represent an average of all control measurements; drug values represent mean values for the peak effect.

Figure 5. Interactions between ethanol and DAMGO in the presence of ryanodine. A1-C1. Normalized mean values ($\pm$ SEM) for the peak amplitude of the K^+ -evoked (60 mM) Ca^{2+} signal under control conditions (vehicle superfusion), during ryanodine superfusion and during superfusion with ryanodine plus DAMGO (A1), ryanodine plus 20 mM ethanol (B1), or ryanodine plus 20 mM ethanol plus DAMGO (C1). In each experiment, the peak amplitude of the Ca^{2+} signal was normalized to the mean value for the peak amplitude during control conditions on an individual cell basis. Horizontal arrows show the time of superfusion of the various drugs. A2-C2. Normalized mean values ($\pm$ SEM) for peak amplitude of the K^+ -evoked Ca^{2+} signals under control conditions (average of all control values) and in the presence of the different drugs tested (t = time point of experiment for data used in graph). Dotted line indicates control values. Asterisk indicates significant difference from control conditions without ryanodine; hatched symbol indicates significantly different from the respective control condition ($p < 0.05$, ANOVA). Ryanodine plus DAMGO, ryanodine plus ethanol, and ryanodine plus ethanol plus DAMGO all reduced the peak amplitude of the Ca^{2+} signal to a similar extent. A3-C3. Mean values ($\pm$ SEM) for time to peak (TTP) and duration (measured at half maximum peak amplitude) of the K^+ -evoked Ca^{2+} signal under control conditions (average of all control values) and in the presence of the different drugs tested at the peak of the drug effect. For all graphs, numbers in bars show the number of cells studied.

Figure 6.

Dynorphin A inhibition of Ca^{2+} signal produced by K^{+} saline in KOR transformed PC12 cells. A. Digitized images of live PC12 cells used for Ca^{2+} imaging studies. The fluorescence image shows expression of KOR-EGFP, which is primarily localized at the cell membrane. B. Representative Ca^{2+} signals evoked by K^{+} saline (50 mM, applied at the arrow) in KOR PC12 cells during control conditions (saline superfusion) and after superfusion of dynorphin A (Dyn A). Responses of 4 cells within a microscopic field are shown. C. Normalized mean peak amplitude ($\pm$ SEM, $n = 27$) of the K^{+} evoked Ca^{2+} signal under control conditions (saline superfusion) and during superfusion of 200 nM Dyn A. In each experiment, the peak amplitude of the Ca^{2+} signal was normalized to the mean value for the peak amplitude during control conditions on an individual cell basis. Horizontal arrows show time of saline (control) and Dyn A superfusion. D. The L-type VGCC blocker nimodipine significantly reduced the Ca^{2+} signal indicating that the primary VGCC responsible for the Ca^{2+} signal was the L-type VGCC. Graph shows mean values ($\pm$ SEM) for the peak amplitude of the K^{+} -evoked Ca^{2+} signal under control conditions and in the presence of nimodipine. E. Normalized mean values ($\pm$ SEM) for peak amplitude of the K^{+} -evoked Ca^{2+} signal under control conditions (at time = -3) and in the presence of 200 nM Dyn A (at $t = 12$ min). Asterisk indicates significant difference from control values; the hatched character indicates significant difference from 2 μM DAMGO ($p < 0.05$, ANOVA). F. Mean values ($\pm$ SEM) for time to peak (TTP) and duration (measured at half maximum peak amplitude) of the K^{+} -evoked Ca^{2+} signal under control conditions and in the presence of 200 nM Dyn A. G. Mean values ($\pm$ SEM) for resting Ca^{2+} levels under control conditions and in the presence of 200 nM Dyn A.

Figure 7.

Interactions between ethanol and Dyn A. A, D. Normalized mean values ($\pm$ SEM) for the peak amplitude of the K^+ -evoked Ca^{2+} signal under control conditions (saline superfusion) and after superfusion of 20 mM ethanol or 20 mM ethanol plus 200 nM Dyn A. In each experiment, the peak amplitude of the Ca^{2+} signal was normalized to the mean value for the peak amplitude during control conditions on an individual cell basis. Horizontal arrows show the time of saline (control), ethanol or ethanol plus Dyn A superfusion. B, E. Representative Ca^{2+} signals evoked by K^+ saline (50 mM, applied at the arrow) in the KOR PC12 cells under control conditions and after superfusion of ethanol (20 mM)(B) or ethanol (20 mM) plus Dyn A (200 nM)(E). Responses of 4 cells within a microscopic field are shown. C, F. Normalized mean values ($\pm$ SEM) for peak amplitude of the K^+ -evoked Ca^{2+} signals under control conditions (at time = -3) and in the presence of 20 mM ethanol (C; at time = 12 min) or 20 mM ethanol plus 200 nM Dyn A (F; at time = 12 min). Ethanol and ethanol plus Dyn A reduced the peak amplitude of the Ca^{2+} signal to a similar extent. Asterisk indicates significant difference from control value ($p < 0.05$, ANOVA). Dotted line indicates control values. For all graphs, numbers in bars show the number of cells studied.