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Differential Effects of Chronic Ethanol Exposure on ATP- and Bradykinin-Induced Increases in Intracellular Calcium Levels in PC-12 Cells

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SUMMARY

The present study investigated the regulation of intracellular calcium levels ($[Ca^{2+}]_i$) by ethanol, ATP, and bradykinin (BK) in PC-12 cells. Acute addition of 150 mM ethanol increased $[Ca^{2+}]_i$ but did not alter ATP- and BK-induced increases in $[Ca^{2+}]_i$. After a 4-day exposure to 150 mM ethanol, the maximal response to ATP was decreased 39.7 ± 8.12% ($p < 0.01$), whereas that to BK was increased 43.8 ± 6.81% ($p < 0.01$). There was no change in the EC_{50} values for either ATP or BK after chronic ethanol exposure. Addition of excess ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) to remove extracellular free calcium prevented the ATP-induced increase in $[Ca^{2+}]_i$. In contrast, BK produced an increase in $[Ca^{2+}]_i$ in the presence of excess EGTA, suggesting that BK releases calcium from internal stores. Consistent with this suggestion, chronic ethanol exposure enhanced BK-induced phosphoinositide hydrolysis. ATP, however, did not increase phosphoinositide hydrolysis. Pretreatment with 10 μM nifedipine, which blocked depolarization-evoked increases in $[Ca^{2+}]_i$, reduced ATP- and BK-induced increases in $[Ca^{2+}]_i$ but did not alter the response to chronic ethanol exposure. Although acute addition of ethanol reduced KCl-stimulated increases in $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ uptake, chronic ethanol exposure did not alter the depolarization-induced increase in $[Ca^{2+}]_i$ or $^{45}Ca^{2+}$ uptake. The present study demonstrates that chronic ethanol exposure inhibits calcium influx through voltage-independent cationic channels associated with purinergic receptors and enhances BK-stimulated phosphoinositide hydrolysis, with a subsequent release of calcium from internal stores.

A variety of neurotransmitters, neuromodulators, and drugs, such as EtOH, affect cellular function through an alteration in $[Ca^{2+}]_i$ (1). Neuronal regulation of $[Ca^{2+}]_i$ has been extensively studied using PC-12 pheochromocytoma cells as a model. In these cells, the neuromodulator/neurotransmitters BK and extracellular ATP have been reported to stimulate $[Ca^{2+}]_i$, but through independent mechanisms (2, 3). The BK stimulation of $[Ca^{2+}]_i$ appears to involve primarily an increase in the generation of $InsP_3$, which causes release of calcium from intracellular stores (2, 4). Although ATP also has been reported to increase $InsP_3$ levels in several PC-12 cell subclones (2, 5), purinergic stimulation of $[Ca^{2+}]_i$ appears to involve mainly an influx of calcium through both ROC and VDCC (2, 6, 7).

EtOH has been shown to alter agonist-stimulated calcium influx and $[Ca^{2+}]_i$, and these changes in calcium levels may be important in the physiological responses to EtOH (8–11). Acute exposure to EtOH was reported to decrease calcium influx through VDCC (12, 13), as well as through ROC regulated by NMDA and kainate (14–16). After chronic treatment with EtOH, depolarization-stimulated calcium influx through VDCC and the density of VDCC were found to be increased in some cell types (12, 13, 17) but not in another (18). Chronic exposure to EtOH also was reported to increase both the number of NMDA receptor-ionophore complexes (19, 20) and NMDA-stimulated calcium influx (18).

With the exception of the NMDA receptor system, there is a paucity of data regarding the neuronal effects of EtOH on receptor-stimulated calcium levels. Therefore, the present study was undertaken to investigate the effects of EtOH on the stimulation of neuronal $[Ca^{2+}]_i$ by ATP and BK. Using PC-12 cells as a neuronal model, these studies demonstrate that chronic EtOH exposure has a differential effect on ATP- and BK-stimulated $[Ca^{2+}]_i$.

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ABBREVIATIONS: EtOH, ethanol; $[Ca^{2+}]_i$, intracellular free calcium level; BK, bradykinin; ROC, receptor-operated cationic channel; VDCC, voltage-dependent calcium channel; NMDA, *N*-methyl-D-aspartate; fura-2/AM, fura-2/acetoxymethyl ester; KRH, Krebs-Ringer-HEPES; InsP, inositol phosphate; $InsP_1$, inositol monophosphate; $InsP_2$, inositol 4,5-bisphosphate; $InsP_3$, inositol 1,4,5-trisphosphate; EC_{50}, concentration of drugs resulting in half-maximal stimulation; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

Experimental Procedures

Materials. The following were used in this study: *myo*-[2-^{3}H]inositol and $^{45}Ca^{2+}$ (DuPont/New England Nuclear, Boston, MA), RPMI 1640 and CMRL 1066 media (GIBCO, Grand Island, NY), fetal calf serum (Hyclone Laboratories, Logan, UT), heat-inactivated horse serum (JRH Bioscience, Lenexa, KS), and fura-2 pentaphosphate and fura-2/AM (Molecular Probes, Eugene, OR). All other chemicals were obtained from standard commercial sources.

Cell culture. PC-12 cells were maintained at 37° on collagen-coated dishes in RPMI 1640 medium supplemented with 5% fetal calf serum and 10% heat-inactivated horse serum, in an atmosphere of 95% air/5% CO_2. Chronic treatment of cells with EtOH was carried out as described previously (21), using plastic desiccators containing an atmosphere of 95% air/5% CO_2 that was saturated with the appropriate concentration of EtOH. For these studies, media were replaced every other day.

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured fluorometrically using the fluorescent calcium indicator fura-2. Briefly, cells were washed twice with KRH buffer (125 mM NaCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 6 mM glucose, 1 mM $CaCl_2$, 25 mM HEPES, pH 7.4) and were harvested by trituration. The cells then were collected by centrifugation at 900 × *g* for 1 min and were incubated for 30 min at 37° in KRH buffer supplemented with 0.2% bovine serum albumin and 2 μM fura-2/AM. The cells were washed three times with KRH buffer and were resuspended (1×10^6 cells/ml) in KRH buffer containing 2.5 mM probenecid. In initial studies, inclusion of 2.5 mM probenecid blocked leakage of the fluorescent dye (<2% leakage over 2 hr) in both control and EtOH-treated groups. Dye leakage was determined by incubating fura-2-loaded cells for 2 hr at room temperature and then measuring the fluorescence intensity of the media using an excitation wavelength of 340 nm and an emission wavelength of 495 nm. Total fura-2 content of the cells was determined by adding 0.1% Triton X-100 to the cell suspension and measuring the fluorescence intensity of the supernatant after centrifugation at 1000 × *g* for 5 min.

Changes in fluorescence intensity of fura-2 at excitation wavelengths of 340 nm and 380 nm were determined at room temperature using a Shimadzu RF-5000 fluorimeter (Shimadzu Co., Kyoto, Japan). $[Ca^{2+}]_i$ was determined as described by Grynkiewicz *et al.* (22) and Ratto *et al.* (23), from the equation $[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)](F_o/F_s)$, where F_o and F_s are the fluorescence intensities of fura-2 pentapotassium salt at an excitation wavelength of 380 nm, using buffered salt solutions containing 20 mM EGTA ($[Ca^{2+}]_{min}$) and 0.1% Triton X-100 ($[Ca^{2+}]_{max}$), respectively, R is the ratio of fluorescence intensities at excitation wavelengths of 340 nm and 380 nm, and R_{max} and R_{min} are values of R determined at the end of each experiment using 0.1% Triton X-100 and 20 mM EGTA, respectively. A K_d value of 224 nM was used (22). Autofluorescence of the cell suspension (observed without fura-2/AM loading into the cells) was 1–3%. At the concentrations used in the present study, none of the reagents significantly contribute to the fluorescence intensities at 340 nm and 380 nm. Photobleaching of the fluorescent dye measured at 360 nm, which is the isosbestic point of fura-2, was negligible over the 10–12 min needed for each test and was not affected by the presence of 150 mM EtOH.

[^{3}H]InsP formation. PC-12 cells on 60-mm culture dishes were preincubated for 18–24 hr with *myo*-[2-^{3}H]inositol (5 μCi/ml) in 1.5 ml of CMRL 1066 medium supplemented with 5% fetal calf serum, 10% heat-inactivated horse serum, 300 mg/liter L-glutamine, and 1 g/liter D-glucose. Cells were washed three times with KRH buffer and were harvested by trituration. Cells were then incubated for 15 min at 37° in KRH buffer containing 10 mM LiCl and 1 mM inositol. Phosphoinositide hydrolysis was measured at 37° in a final volume of 650 μl containing KRH, 10 mM LiCl, 1 mM inositol, and appropriate drugs. The reaction was terminated after 5 min with 500 μl of ice-cold 9% perchloric acid, and samples were incubated on ice for 15 min. The perchloric acid extract was sonicated for 2 min and then centrifuged at 1000 × *g* for 10 min. The resulting pellets were solubilized with 0.1 N NaOH and used for the determination of protein content by the method of Lowry *et al.* (24). The supernatants were neutralized with a solution containing 0.5 M KOH, 9 mM $Na_2B_4O_7$, and 1.9 mM EDTA, and then the [^{3}H]InsPs were separated by anion exchange chromatography on Dowex columns as described previously (25). Briefly, the neutralized supernatants were added to 0.75-ml columns of Dowex 1-X8 resin (100–200 mesh, formate form; Bio-Rad). $InsP_1$ was eluted with 0.2 M ammonium formate plus 0.1 M formic acid. The more polar $InsP_2$ and $InsP_3$ were eluted with 0.4 M ammonium formate plus 0.1 M formic acid and 1 M ammonium formate plus 0.1 M formic acid, respectively. Radioactivity in each fraction was determined by liquid scintillation counting. For the measurement of total [^{3}H]inositol labeling of phospholipids, cells were washed three times with KRH buffer and were harvested by trituration. Cells were extracted twice with chloroform/methanol (2:1) for 10 min at room temperature, and the amount of radioactivity in the combined chloroform layers was determined by liquid scintillation counting.

Measurement of $^{45}Ca^{2+}$ uptake. $^{45}Ca^{2+}$ uptake was measured using cells attached to the tissue culture plates as well as cells in suspension. For the measurement of $^{45}Ca^{2+}$ uptake into cells in suspension, PC-12 cells were washed three times with KRH buffer and were harvested by trituration. $^{45}Ca^{2+}$ uptake was measured at room temperature using 0.5 ml of KRH containing 1 μCi of $^{45}Ca^{2+}$. Depolarization-evoked $^{45}Ca^{2+}$ uptake was measured using KRH containing 50 mM KCl. After 2.5 min, uptake was terminated by vacuum filtration using Whatman GF/B glass fiber filters, and the filters were washed three times with 2.5 ml of ice-cold KRH buffer supplemented with 5 mM $LaCl_3$ to block nonspecific binding of $^{45}Ca^{2+}$. Radioactivity in 0.1% Triton X-100 cell digests was determined by liquid scintillation counting.

For the measurement of $^{45}Ca^{2+}$ uptake into PC-12 cells attached to 35-mm culture plates, cells were rinsed twice with 1.5 ml of a HEPES-buffered solution (buffer A, 85 mM NaCl, 45 mM choline chloride, 5 mM KCl, 1 mM $CaCl_2$, 5 mM glucose, 50 mM HEPES, pH 7.4) and were preincubated for 20 min at room temperature in 1.5 ml of fresh buffer. $^{45}Ca^{2+}$ uptake was measured at room temperature in 0.6 ml of buffer A containing 1 μCi of $^{45}Ca^{2+}$. For the measurement of depolarization-evoked $^{45}Ca^{2+}$ uptake, 50 mM KCl was isotonically substituted for choline chloride. After 2.5 min at 25°, medium was rapidly removed and the cells were washed three times with 1.5 ml of ice-cold buffer A. Radioactivity in neutralized 0.5 N NaOH cell digests was determined by liquid scintilation counting.

[^{3}H]PN200–110 binding assay. Cells were washed three times with KRH buffer and were harvested by trituration. Cells were then homogenized in 20 volumes of 50 mM Tris·HCl, pH 7.5, using a Teflon pestle, and the homogenate was centrifuged at 45,000 × *g* for 45 min at 4°. Aliquots of the resuspended pellet in 50 mM Tris·HCl (200–300 μg of protein) were incubated at 37° for 2.5 hr in 50 mM Tris·HCl, pH 7.2, containing [^{3}H]PN200–110 (7.5×10^{-12} to 5×10^{-10} M), in the absence and presence of 100 μM unlabeled PN200–110 to define nonspecific binding. Incubations were terminated by vacuum filtration using GF/B glass fiber filters, and the filters were rinsed twice with 5 ml of ice-cold 50 mM Tris·HCl, pH 7.2. Radioactivity on filters was measured by liquid scintillation counting.

Data analyses and statistics. Data are presented as mean ± standard error of the mean and were analyzed for statistical significance using a paired *t* test. The concentration-response relationship was analyzed using a standard set of pharmacological programs (26). Radioligand binding data were analyzed with the program EBDA/LIGAND (Elsevier BIOSOFT).

Results

ATP, BK, and EtOH each produced biphasic increases in $[Ca^{2+}]_i$ (Fig. 1). The EtOH-induced increase in $[Ca^{2+}]_i$ was not an osmotic effect, because the addition of 150 mM choline chloride did not change $[Ca^{2+}]_i$ (data not shown). Although the acute addition of 150 mM EtOH increased the peak $[Ca^{2+}]_i$ stimulated by ATP, this increase was due to the ability of EtOH

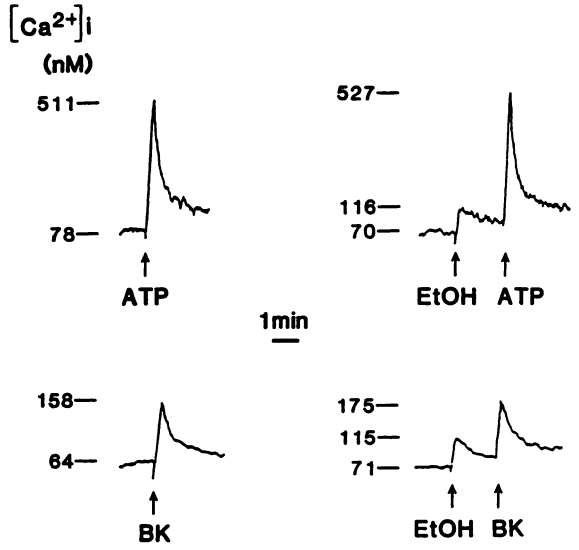

Fig. 1. Acute effects of EtOH on ATP (100 μM)- and BK (100 nM)-stimulated increases in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured in the absence and presence of 150 mM EtOH. *Traces* are representative of five to eight separate experiments.

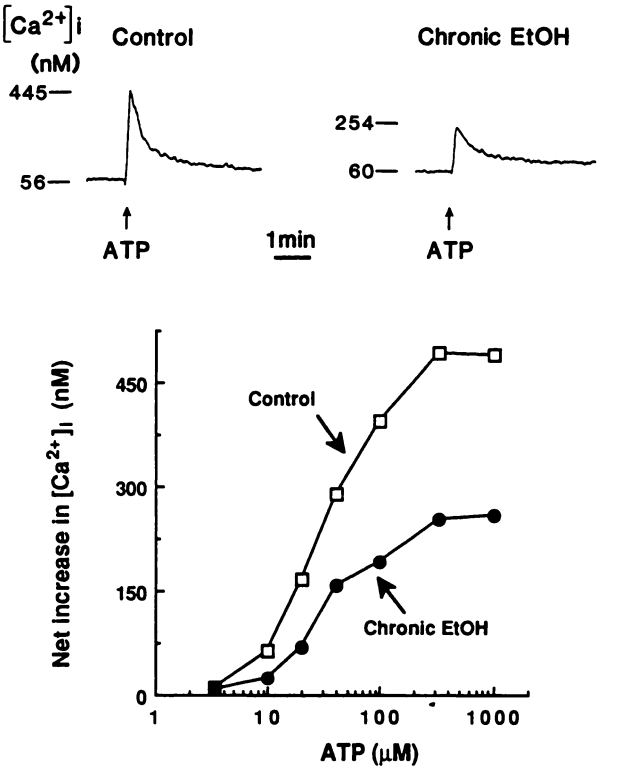

Fig. 2. Reduction in ATP-stimulated $[Ca^{2+}]_i$ after chronic EtOH exposure. The PC-12 cells were treated for 4–5 days with 150 mM EtOH, as described in Experimental Procedures. *Upper*, representative *traces* of $[Ca^{2+}]_i$ stimulated by 100 μM ATP; *lower*, concentration-response relationship for ATP-induced increases in $[Ca^{2+}]_i$. Data are expressed as the net increase in peak $[Ca^{2+}]_i$ stimulated by ATP and are representative of four separate experiments.

alone to raise $[Ca^{2+}]_i$. After correction for this EtOH-induced increase in basal $[Ca^{2+}]_i$, the presence of EtOH did not alter the net increase in peak $[Ca^{2+}]_i$ stimulated by 100 μM ATP (408 ± 23.0 nM and 424 ± 29.5 nM for cells in the absence and presence of EtOH, respectively; seven experiments). Similarly, the BK-induced increase in peak $[Ca^{2+}]_i$ was not altered by the presence of EtOH (85.1 ± 9.08 nM and 79.9 ± 5.17 nM for cells in the absence and presence of EtOH, respectively; five experiments).

The resting $[Ca^{2+}]_i$ was not altered by a 4-day exposure to 150 mM EtOH. In control cells $[Ca^{2+}]_i$ was 78.6 ± 5.90 nM (12 experiments), whereas in EtOH-treated cells $[Ca^{2+}]_i$ was 87.0 ± 4.13 nM (eight experiments). Chronic EtOH treatment decreased the maximal response to ATP by 40% (net increase in $[Ca^{2+}]_i$ was 447 ± 34.8 nM for control cells and 269 ± 18.3 nM for EtOH-treated cells; $p < 0.01$; four experiments) (Fig. 2). In contrast, the maximal response to BK was enhanced 44% by chronic EtOH treatment (net increase in $[Ca^{2+}]_i$ was 88.7 ± 3.18 nM for control cells and 129 ± 3.51 nM for EtOH-treated cells; $p < 0.01$; five experiments) (Fig. 3). Chronic EtOH exposure did not alter the EC_{50} for either ATP (31.4 ± 4.29 nM and 29.5 ± 4.13 nM for control and EtOH-treated cells, respectively; four experiments) or BK (3.01 ± 0.37 nM and 4.01 ± 0.59 nM for control and EtOH-treated cells, respectively; five experiments).

The contribution of extracellular calcium in agonist-stimulated $[Ca^{2+}]_i$ was investigated by adding 6 mM EGTA to the cell suspension. Addition of excess EGTA rapidly decreased basal $[Ca^{2+}]_i$ (Fig. 4). A similar finding has been reported in platelets and neutrophils (27) and appears to be due to a rapid exchange of calcium ions through the cell membrane. Consistent with this suggestion, addition of 2 mM calcium resulted in a rapid and sustained increase in basal $[Ca^{2+}]_i$ of 44.1 ± 1.28 nM (three experiments). In the presence of excess EGTA, ATP did not alter $[Ca^{2+}]_i$ (Fig. 4, *upper*). A $Mg \cdot ATP^{2-}$ complex is the ligand for some of the P_2 subtypes of purinergic receptors, but addition of Mg^{2+} in excess of the EGTA did not restore the ATP response (data not shown). Thus, the lack of an ATP response was not due to chelation of free extracellular Mg^{2+}. In the presence of excess EGTA, BK produced a rapid but transient increase in $[Ca^{2+}]_i$ (Fig. 4, *lower*). The increase in peak $[Ca^{2+}]_i$ measured in the presence of EGTA was enhanced 115% after chronic EtOH treatment (net increase in BK-stimulated $[Ca^{2+}]_i$ was 35.2 ± 3.81 nM and 75.8 ± 8.12 nM in control and EtOH-treated cells, respectively; $p < 0.001$; five experiments).

Additional studies were undertaken to investigate whether the EtOH-induced increase in BK-stimulated $[Ca^{2+}]_i$ involved an increase in InsP formation. BK increased the generation of InsPs (Fig. 5) and specifically increased $InsP_3$ formation by 78.3 ± 25.2% ($InsP_3$ levels were 527 ± 50.5 dpm/mg of protein in control cells and 940 ± 71.5 dpm/mg of protein in BK-stimulated cells). After chronic EtOH exposure, basal levels of phosphoinositide hydrolysis were increased 28.6 ± 5.31%, whereas the stimulatory effect of BK on InsP generation was increased 113 ± 23.7%. Similarly, chronic EtOH treatment increased basal $InsP_3$ formation to 704 ± 61.9 dpm/mg of protein, whereas BK-stimulated $InsP_3$ formation was increased to 1719 ± 83.4 dpm/mg of protein. Chronic EtOH exposure, however, did not alter inositol lipid labeling; incorporation of [^{3}H]inositol into phospholipids was 104,501 ± 5,072 dpm/mg of protein in control cells and 99,197 ± 6,893 dpm/mg of protein in EtOH-treated cells. The changes described above were not due to residual EtOH from the chronic treatment. Although the addition of 150 mM EtOH increased basal InsP generation by 13.6%, acute addition of EtOH decreased BK stimulation of InsP formation by 50.4 ± 4.23% (Fig. 6). Addition of ATP (100 μM) did not alter generation of InsPs (data not shown).

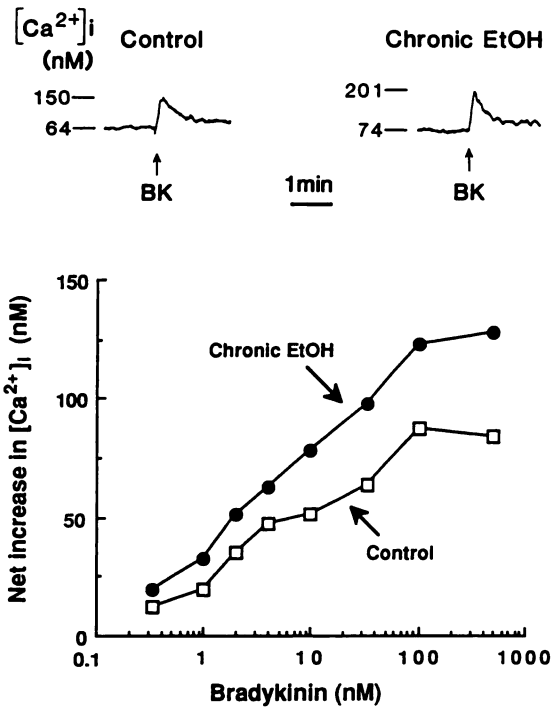

Fig. 3. Enhancement of BK-stimulated $[Ca^{2+}]_i$ after chronic EtOH exposure. The PC-12 cells were treated for 4–5 days with 150 mM EtOH, as described in Experimental Procedures. *Upper*, representative *traces* of $[Ca^{2+}]_i$ stimulated by 100 nM BK; *lower*, concentration-response relationship for BK-induced increases in $[Ca^{2+}]_i$. Data are expressed as the net increase in peak $[Ca^{2+}]_i$ stimulated by BK and are representative of five separate experiments.

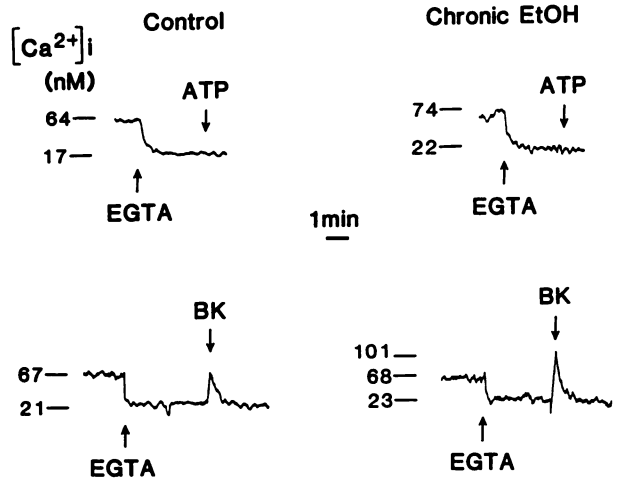

Fig. 4. Effects of EGTA on ATP- and BK-induced increases in $[Ca^{2+}]_i$. After a 4-day exposure to 150 mM EtOH, ATP (100 μM)- and BK (100 nM)-induced increases in $[Ca^{2+}]_i$ were measured in the presence of 6 mM EGTA. In the presence of excess EGTA, the extracellular level of free calcium was <90 nM, as measured using the cell membrane-impermeable fluorescent dye fura-2 pentaphosphate. *Traces* are representative of five separate experiments.

The data described above indicate that, although the acute addition of EtOH inhibits BK stimulation of $InsP_3$ formation, there appears to be no corresponding reduction in calcium release, inasmuch as the BK-stimulated rise in $[Ca^{2+}]_i$ was not altered by the inclusion of EtOH (see Fig. 1). This apparent difference was further investigated by determining the effects of acute EtOH on $[Ca^{2+}]_i$ in the absence of extracellular free

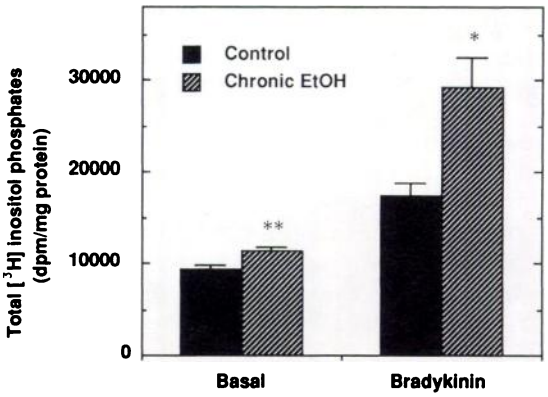

Fig. 5. Effects of chronic exposure to EtOH on phosphoinositide hydrolysis. Stimulation of InsP formation by 100 nM BK was determined in control cells and cells treated with 150 mM EtOH for 4 days. Data are expressed as the sum of $InsP_1$, $InsP_2$, and $InsP_3$ and are plotted as mean ± standard error from five separate experiments. *, $p < 0.05$; **, $p < 0.01$, compared with control.

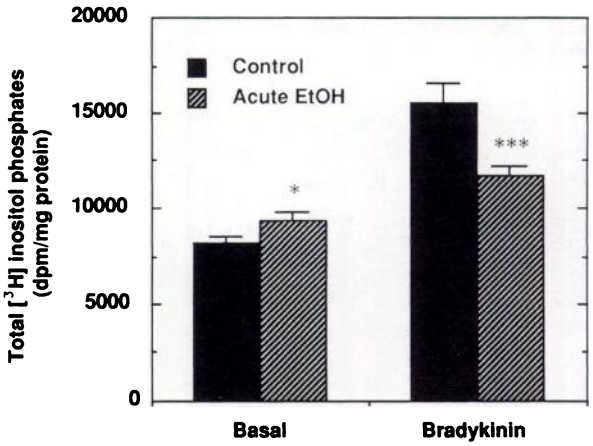

Fig. 6. Effects of acute EtOH treatment on phosphoinositide hydrolysis. BK (100 nM) stimulation of InsP formation was measured in the absence and presence of 150 mM EtOH *in vitro*. Data are expressed as the sum of $InsP_1$, $InsP_2$, and $InsP_3$ and are plotted as mean ± standard error from five separate experiments. *, $p < 0.05$; ***, $p < 0.001$, compared with control.

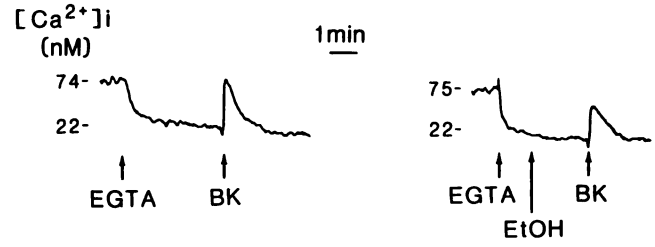

Fig. 7. Acute effects of EtOH on BK (100 nM)-stimulated increases in $[Ca^{2+}]_i$ in the absence of extracellular free Ca^{2+}. $[Ca^{2+}]_i$ was measured in the absence and presence of 150 mM EtOH. Extracellular free Ca^{2+} was removed by the addition of excess (6 mM) EGTA. *Traces* are representative of four separate experiments.

calcium. In this situation BK stimulation of calcium influx would be prevented and only BK-stimulated release of internal calcium stores would be measured. In the presence of EGTA, the acute addition of EtOH did not alter basal $[Ca^{2+}]_i$ (Fig. 7). However, in the absence of extracellular free calcium, the inclusion of EtOH significantly ($p < 0.05$) reduced the BK-stimulated increase in $[Ca^{2+}]_i$, by 36.9 ± 8.19% (Fig. 7).

The role of VDCC in the response of PC-12 cells to chronic

EtOH was investigated using the dihydropyridine nifedipine. Addition of 10 μM nifedipine blocked the 50 mM K^+-induced $[Ca^{2+}]_i$ rise by 81%; higher concentration of nifedipine did not further block the KCl response. In control cells, the ATP-induced increase in $[Ca^{2+}]_i$ was suppressed 31% in the presence of nifedipine (Fig. 8). However, EtOH-induced inhibition of the ATP response was not altered by nifedipine; chronic EtOH exposure decreased ATP-stimulated $[Ca^{2+}]_i$ by 34.4 ± 6.21% and 29.8 ± 7.4% in the absence and presence of nifedipine, respectively (five experiments). Similarly, nifedipine reduced BK-stimulated $[Ca^{2+}]_i$ by 24% (Fig. 8), but the potentiating effect of chronic EtOH exposure on BK-stimulated $[Ca^{2+}]_i$ was not changed; chronic EtOH exposure increased BK-induced $[Ca^{2+}]_i$ by 28.9 ± 4.02% and 34.5 ± 3.33% in the absence and presence of nifedipine, respectively (five experiments).

The data described above appear to indicate that calcium influx through VDCC was not altered by the chronic EtOH exposure. Because chronic EtOH exposure has been reported to alter VDCC (12, 13, 17), additional studies were undertaken to determine the effects of chronic EtOH treatment on $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ uptake stimulated by high K^+. In initial studies maximum depolarization-dependent increases in $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ uptake were obtained using 50 mM KCl (data not shown). The addition of 150 mM EtOH *in vitro* reduced KCl (50 mM) stimulation of both $[Ca^{2+}]_i$ (55.1 ± 3.65%; $p < 0.001$; five experiments) and $^{45}Ca^{2+}$ uptake (34.2 ± 4.98%; $p < 0.05$; five experiments). However, a 4-day treatment of PC-12 cells with 150 mM EtOH did not alter the depolarization-induced increase in $[Ca^{2+}]_i$ (net increase in $[Ca^{2+}]_i$ was 178 ± 14.2 nM and 172 ± 9.27 nM for control and EtOH-treated cells, respectively; five experiments). Similarly, a 4-day exposure of PC-12 cells to EtOH did not alter KCl-induced $^{45}Ca^{2+}$ uptake measured in cells in suspension (data not shown) or in cells attached to tissue culture plates (Fig. 9). Furthermore, a 7-day treatment with 150 mM EtOH also did not alter net uptake of $^{45}Ca^{2+}$ stimulated by 50 mM KCl (Fig. 9). In addition, a 6-day exposure of PC-12 cells to 150 mM EtOH did not alter either the density of dihydropyridine binding sites (B_{max} was 61.6 ± 10.2 fmol/mg of protein and 73.8 ± 10.1 fmol/mg of protein for control and

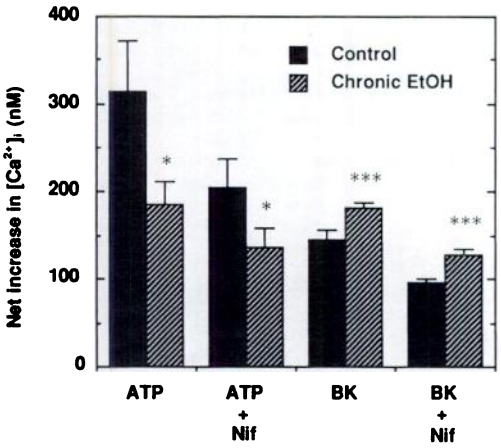

Fig. 8. ATP- and BK-induced $[Ca^{2+}]_i$ rise in the presence of 10 μM nifedipine (*Nif*). After a 4-day exposure to 150 mM EtOH, ATP (100 μM)- and BK (100 nM)-induced increases in $[Ca^{2+}]_i$ were measured in the absence and presence of 10 μM nifedipine. Nifedipine was added 1–2 min before addition of ATP or BK. Data are expressed as mean ± standard error from five separate experiments. *, $p < 0.05$; ***, $p < 0.001$, compared with control.

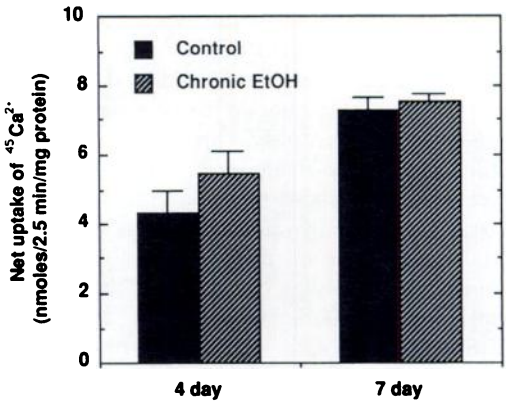

Fig. 9. Chronic EtOH effects on depolarization-evoked $^{45}Ca^{2+}$ uptake. The PC-12 cells were treated for 4 or 7 days with 150 mM EtOH. $^{45}Ca^{2+}$ uptake was measured using PC-12 cells attached to the plates, as described in Experimental Procedures. Data are expressed as mean ± standard error from 12 (4-day EtOH treatment) or five (7-day EtOH treatment) separate experiments.

EtOH-treated cells, respectively; four experiments) or the affinity of the sites for [^{3}H]PN200–110 (K_d was 86.2 ± 21.0 pM and 119 ± 39.4 pM for control and EtOH-treated cells, respectively; four experiments).

Discussion

In the PC-12 cells acute addition of EtOH, ATP, and BK all increased $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ observed after the acute addition of EtOH was dependent on an influx of calcium, inasmuch as removal of extracellular free calcium prevented the EtOH-induced rise in basal $[Ca^{2+}]_i$. The presence of EtOH, however, did not alter the ATP-stimulated rise in $[Ca^{2+}]_i$. Thus, compared with the NMDA, γ-aminobutyric acid, and 5-hydroxytryptamine$_3$ subtype ROC (14, 28, 29), the ATP ROC appears more resistant to the effects of acute EtOH. Acute addition of EtOH inhibited BK-stimulated $InsP_3$ formation, but the expected reduction in the BK-stimulated $[Ca^{2+}]_i$ was only observed in the absence of extracellular free calcium. In normal KRH buffer, any reduction in calcium release from internal stores due to the inhibitory action of acute EtOH treatment on BK-stimulated $InsP_3$ formation appears to have been counterbalanced by an EtOH-induced increase in calcium influx.

The ATP-induced increase in $[Ca^{2+}]_i$ was due to an influx of extracellular Ca^{2+}, because inclusion of excess EGTA prevented the ATP response. Furthermore, experiments using nifedipine to block dihydropyridine-sensitive VDCC indicate that ATP-induced Ca^{2+} influx occurs primarily through ROC (69.3%) but also contains a contribution from VDCC. After chronic exposure to EtOH, the maximum response to ATP was reduced, without any alteration in EC_{50}. This inhibitory effect of chronic EtOH exposure on ATP-stimulated $[Ca^{2+}]_i$ is due solely to a change in the ROC, because nifedipine did not alter the response elicited by the chronic treatment. Although the ATP ROC appears to be resistant to the acute actions of EtOH, this ROC appears to be very sensitive to chronic EtOH exposure, because preliminary results indicate a significant reduction in the ATP-stimulated increase in $[Ca^{2+}]_i$ after chronic exposure to 25 mM EtOH.[1] This alcohol concentration is comparable to

[1] W.-K. Kim and R. A. Rabin, unpublished observations.

the legal limit for driving while intoxicated. Chronic EtOH exposure also has been reported to alter other ROC. For example, NMDA-stimulated calcium influx and the density of NMDA-ionophore complex were increased after chronic EtOH exposure (18–20, 30). Conversely, chronic EtOH exposure reduced γ-aminobutyric acid-stimulated chloride influx (28, 29).

In other cells ATP has been reported to increase $[Ca^{2+}]_i$, at least in part, by an $InsP_3$-stimulated release of intracellular Ca^{2+} (31–34). Such a mechanism is not operable in the PC-12 cells used in the present study, because ATP did not stimulate $InsP_3$ formation. This difference may be due to expression of different purinergic P_2 receptor subtypes. For example, the P_{2z} subtype is not linked to phospholipase C (35).

The BK-stimulated increase in $[Ca^{2+}]_i$ occurs through both release of intracellular Ca^{2+} by $InsP_3$ and influx of extracellular Ca^{2+}. Existence of this influx is shown by the reduction in the BK-induced increase in $[Ca^{2+}]_i$ after the inclusion of EGTA and nifedipine. In contrast to the response to ATP, chronic EtOH treatment enhanced BK stimulation of $[Ca^{2+}]_i$. The EtOH-induced potentiation of BK-stimulated $[Ca^{2+}]_i$ appears to involve only the mobilization of intracellular Ca^{2+}. Thus, EtOH-induced potentiation of the BK response was not affected by removal of extracellular Ca^{2+} or by inclusion of nifedipine. In addition, chronic EtOH treatment increased phosphoinositide hydrolysis in response to BK stimulation. However, the effects of chronic EtOH on the activation of phospholipase C are complex and appear to be cell specific. Chronic treatment of primary astrocyte cultures with 50 mM EtOH also enhanced stimulation of InsPs by norepinephrine (36), but BK stimulation of InsPs was reduced in NG108–15 neuroblastoma-glioma hybrid cells after chronic EtOH exposure (37). Chronic exposure to EtOH also decreased BK stimulation of InsP levels in N1E-115 neuroblastoma cells, but stimulation of InsPs by neurotensin in these cells was unchanged (38). Furthermore, stimulation of phosphoinositide hydrolysis by GTP analogues was reduced in NG108–15 cells (39) but not in N1E-115 cells after chronic EtOH exposure (38).

In the present study, *in vitro* EtOH exposure decreased depolarization-stimulated calcium influx through VDCC. In contrast to previous reports (12, 13, 17), however, chronic exposure to EtOH did not alter the function or density of L-type calcium channels. Thus, no changes in depolarization-stimulated $[Ca^{2+}]_i$, in the influx of $^{45}Ca^{2+}$ either in cell suspension or in cells attached to plates, or in the binding characteristics of the dihydropyridine [^{3}H]PN200–110 were observed after chronic EtOH treatment. The reason for the difference between the present data and previous reports (12, 13, 17) is unclear but may be explained by the use of different subclones of PC-12 cells. However, chronic EtOH exposure of cerebellar granule cells also was reported not to alter Ca^{2+} influx through L-type calcium channels (18). Thus, it appears that EtOH-induced changes in VDCC are not a simple adaptation to inhibition of VDCC function.

In summary, chronic exposure of PC-12 cells to EtOH has opposite effects on ATP- and BK-induced increases in $[Ca^{2+}]_i$. Chronic EtOH exposure produces a selective decrease in the functioning of the ROC regulated by ATP. The selective enhancement of BK-stimulated intracellular calcium release after chronic EtOH exposure involves an increased activation of phospholipase C.

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