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Ethanol Activates Maxi Ca^{2+}-activated K^+ Channels of Clonal Pituitary (GH3) Cells

M. Jakab, T.M. Weiger, A. Hermann

Department of Physiology, Institute of Zoology, University of Salzburg, Hellbrunnerstr. 34, A-5020 Salzburg, Austria

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Abstract. The effect of ethanol on maxi Ca^{2+}-activated K^+ channels (BK channels) in GH3 pituitary tumor cells was investigated using single-channel recordings and focusing on intracellular signal transduction. In outside-out patches, ethanol caused a transient concentration-dependent increase of BK-channel activity. 30 mM (1.4‰) ethanol significantly increased mean channel open time and channel open probability by 26.3 ± 9% and 78.8 ± 10%, respectively; single-channel current amplitude was not affected by ethanol. The augmenting effect of ethanol was blocked in the presence of protein kinase C (PKC) inhibitors staurosporine, bisindolylmaleimide, and PKC (19–31) pseudosubstrate inhibitor as well as by AMP-PNP (5′-adenylylimidodiphosphate), a nonhydrolyzable ATP-analogue, but not by the phospholipase C blocker U-73122. Phosphatase inhibitors microcystin-LR and okadaic acid promoted the ethanol effect. The blocking effect was released at higher concentrations of ethanol (100 mM) suggesting a second site of action or a competition between blockers and ethanol. Our results suggest that the effect of ethanol on BK-channels is mediated by PKC stimulation and phosphorylation of the channels which increases channel activity and hence may influence action potentials duration and hormone secretion.

Key words: Alcohol — Ethanol — BK channel — Pituitary tumor cells — Channel phosphorylation — Protein kinase C (PKC) — GH3 cells

Introduction

Maxi Ca^{2+}-activated K^+ channels (BK channels) are found in a large variety of animal cells including neurons, glandular or epithelial tissue, smooth muscle cells, and excitable endocrine cells. They play an important role in the regulation of neuronal excitability, cell volume regulation, excitation-contraction coupling, and hormonal secretion (Hermann & Hartung, 1983; Petersen & Maruyama, 1984; Marty, 1989). BK channels are activated by voltage together with a rise of the intracellular calcium concentration (McManus, 1991) and, in addition, are regulated via phosphorylation and dephosphorylation processes (Gray et al., 1990; Reinhart et al., 1991; Reinhart & Levitan, 1995).

Excitable endocrine cells, such as pituitary tumor (GH3) cells, which are known to produce growth hormone (GH) and prolactin (Stojilkovic & Catt, 1992; Tashjian, Bancroft & Levine, 1970) exhibit spontaneous repetitive Ca^{2+}-dependent action potentials. The duration and generation of these action potentials is controlled by BK-channel activity thus regulating hormone secretion (Simasko, 1994). Prepubertal exposure of female rats to ethanol has been shown to reduce plasma growth hormone (GH) levels (Dees et al., 1990), and Valimaki et al. (1990) reported a suppression of pulsatile growth hormone release in men after ethanol ingestion. Furthermore, GH-levels in adult male rats were found to be reduced at low ethanol plasma concentrations (30 mM) whereas at higher concentrations of ethanol pulsatile GH-secretion was completely suppressed (Fernstorm, Parkinson & Ebaugh, 1995). It is still poorly understood if these changes are due to hypothalamic perturbations or take place at the pituitary level by changing the secretory behavior of somatotrophs.

Several investigators found that ethanol in physiologically relevant concentrations influences Ca^{2+}-activated K^+ activity in different preparations (Carlen, Gurevich & Durand, 1982; Madsen & Edeson, 1990; Dopico, Lemos & Treistman, 1996). In rat hippocampus CA1 neurons it was shown that ethanol augmented the long-lasting Ca^{2+}-mediated after-hyperpolarization (AHP) indicating an increase of a Ca^{2+}-activated K^+ con-

Correspondence to: A. Hermann

ductance (Carlen et al., 1982). Ethanol applied to mollusc neurons resulted in a depression of both action potential duration and frequency by fastening the repolarization and enhancing the AHP due to activation of Ca^{2+}-activated K^+ current (Madsen & Edeson, 1990). Recently, Dopico et al. (1996) described a sustained, reversible activation of BK-channels by ethanol in excised patches from neurohypophyseal terminals that could explain reduced vasopressin and oxytocin release from the neurohypophysis. The authors suggested channel activation either to be caused by direct interaction of ethanol with the channel protein, or with so far unknown membrane-bound mediators. The mechanisms by which ethanol modulates BK-channel activity have not been determined, however.

We recently reported that ethanol transiently elevates intracellular Ca^{2+} concentration in rat hippocampal neurons along with activation of protein kinase C (PKC), which both may be involved in cytoskeletal rearrangements (Mironov & Hermann, 1996). In this preparation ethanol also appears to affect synaptic transmission involving the activation of a G-protein/PKC mechanism (Lahnsteiner & Hermann, 1995). The aim of the present study was to further elucidate the effects of ethanol and Ca^{2+} on the level of single ion channel activity. We used BK channels from a clonal pituitary cell line to investigate their interaction with ethanol and its effect on phosphorylation processes, which may be of importance in the regulation of hormone secretion in GH producing cells. A preliminary report in abstract form has been published elsewhere (Jakab, Weiger & Hermann, 1996).

Materials and Methods

Cell Culture

GH3 cells were cultured as described previously (Weiger & Hermann, 1994). In brief, GH3 cells from passage 44 to 58 were grown in Minimal Essential Medium (MEM-Earle; Seromed) supplemented with 7% fetal calf serum, 3% horse serum in an atmosphere with 90% humidity and 5% CO_2 at 37°C. For experiments cells were grown on poly-D-lysin coated coverslips and used for recordings 2–3 days after splitting.

Solutions

The bath solution contained (in mM): 145 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 10 glucose, pH 7.3., and the pipette solution in mM: 140 KCl, 2 $MgCl_2$, 20 HEPES, 20 glucose, 1 EGTA, 0.88 $CaCl_2$, with the pH adjusted to 7.2. The free Ca^{2+} concentration was 1×10^{-6} M as calculated with Eqcal software (Biosoft, UK) and measured with a Ca^{2+}-sensitive electrode. Stock solutions of bisindolylmaleimide (Boehringer-Mannheim), staurosporine (Sigma) and U-73122 (Research Biochemicals International) were prepared in DMSO (dimethylsulfoxide) and diluted with bath solution to give a final concentration of 1 μM. Microcystin-LR (Biomol), dissolved in DMSO and okadaic acid (Research Biochemicals International), dissolved in water, were added to the pipette solution to give a concentration of 0.25 μM and 0.6 μM, respectively. Maximum final DMSO concentrations were ≤0.5%. Protein kinase C (19–31) pseudosubstrate was from Biomol and AMP-PNP (5′-adenylylimidodiphosphate) from Sigma. Ethanol solutions were prepared by adding the appropriate amount of ethanol to the bathing solution. For rapid solution exchange (effective exchange rates were less than 1 sec as determined with tetraethylammonium as channel blocker), membrane patches were held in a stream of test solution from a second pipette. To avoid unspecific changes of channel activity according to solution flow, patches were continuously perfused.

Because of the volatile character of ethanol, its actual concentration in the recording chamber was controlled using an ethanol determination kit from Boehringer-Mannheim (≠176290). In solutions containing 30 and 100 mM ethanol, concentrations were found not to be reduced even after remaining in the perfusion system for 4 hr (33.2 ± 1.3 mM and 103.4 ± 2.3 mM after 4 hr; $n = 3$).

Osmolarity of experimental solutions was controlled with a Roebling Automatic Osmometer (Roebling Meßtechnik, Berlin, FRG). The osmolarity in normal solution was 318, after addition of 30 mM ethanol or sucrose, 345 and 337 mosmol/l, respectively. The difference between ethanol and sucrose containing solution was 2 mosmol/l; in the case of 100 mM ethanol or sucrose solutions the difference was 18 mosmol/l.

Electrophysiology

Single-channel recordings were performed at room temperature (20–22°C). Outside-out and inside-out patches were formed (Hamill et al., 1981) and held at a holding potential of +30 mV. Patch pipettes were manufactured from borosilicate glass capillaries (Clark Electromedical Instruments, UK) and had tip resistances of 5–10 MΩ. Signals were amplified (List EPC-7), low-pass filtered at 3 kHz and stored on digital tape (DTR-1200/Biologic). Data were sampled in portions of 12 sec 1–2 min after formation of excised patches after stable conditions were reached for control recordings. 5–10 sec after switching to ethanol-containing solution further recordings were made. In experiments where the time course of the ethanol effect was studied, continuous channel activity over 1 min or more was recorded. Each patch served as its own control. Records were further filtered at 1 kHz and analyzed with pClamp software (Axon Instruments, Foster City, CA). For detection of open channels the 50%-threshold method was used. Amplitude histograms were fitted to a Gaussian distribution. Average channel open probability P_o in multi-channel patches is given by the sum from $i = 1 - N$ of $(t_i \cdot i/t_{tot} \cdot N)$, t_i = the total time during the recording when i channels are open, t_{tot} = total recording time, and N = number of individual channels detected in the patch.

Statistics

Each experiment was repeated at least three times and significance levels of means were determined using Student's *t*-test or Mann-Whitney's test where appropriate with the level of significance set at $P < 0.01$ or $P < 0.05$. Each patch served as its own control. Measurements are expressed as mean ± SEM; controls were set to 100% (= normalized).

Results

After forming outside-out or inside-out patches, BK channels, as described and characterized previously in GH3 cells (Weiger & Hermann, 1994) were observed.

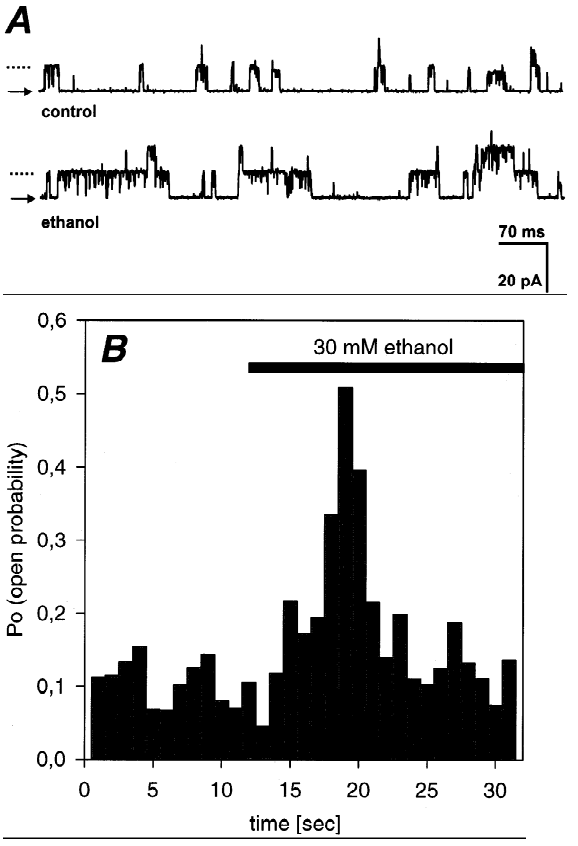

Fig. 1. Action of ethanol on Ca^{2+}-activated K^+ (BK)-channel activity. (*A*) BK-channel activity before (control) and after ethanol (30 mM) application. Outside-out patch; holding potential +30 mV. Note, the increase of channel activity and mean open time of channels. Arrows indicate the closed state of the channel, dots indicate the first open level. (*B*) Plot of channel open probability vs. time. Application of ethanol (30 mM) is followed by a transient increase of channel activity. The time of ethanol application is indicated by the horizontal bar. Each vertical bar represents the average of a 1-sec interval. Outside-out patch; holding potential +30 mV.

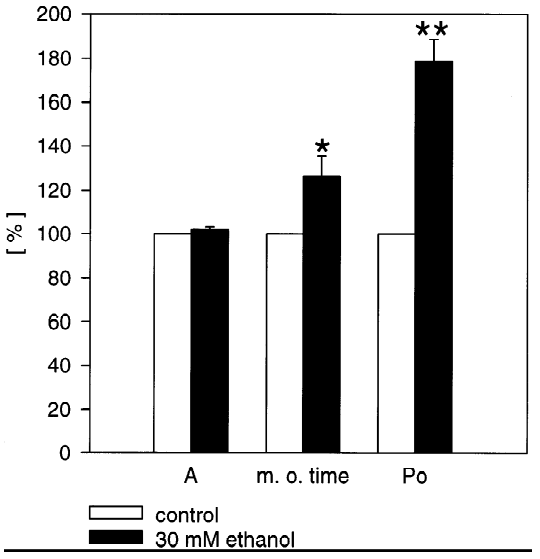

Fig. 2. Ethanol effects on single-channel parameters. Effect of 30 mM ethanol on single current amplitude (*A*), mean channel open time (m.o. time) and channel open probability (P_o). Holding potential +30 mV; normalized data ($n = 13$); *)$P < 0.05$, **)$P < 0.01$.

Application of 30 mM (1.4‰) ethanol to excised outside-out patches resulted in an increase of BK-channel activity (Fig. 1, 2). The mean channel open time and the single-channel open probability were significantly increased by 26.3 ± 9% and by 78.8 ± 10% ($n = 13$), respectively. The single-channel current amplitude was not affected by these ethanol concentrations (Fig. 1, 2). The augmenting effect of ethanol occurred with a delay of 7.5 ± 0.7 sec ($n = 6$) during continuous perfusion with experimental ethanol solution. After about 30 sec, both the mean channel open time and the open probability returned to control values (Table). Addition of 1 mM ATP to the pipette solution or application of higher ethanol concentrations (300 mM) did not significantly change the transient character of the ethanol effect. The onset of the effect, however, occurred significantly earlier in the case of 300 mM (4.0 ± 0.7 sec; $n = 6$). In contrast, addition of 5 mM AMP-PNP, a nonhydrolyzable ATP analogue, to the pipette solution prevented the action of 30 mM ethanol (Table).

The activation of BK channels by ethanol was found to be concentration dependent from 1 to 300 mM, a range reported by Diamond (1992) to be physiologically relevant in chronic alcohol abusers, with a half maximal response (EC_{50} value) at 65 mM (at V = +30 mV) (Fig. 3). A Hill slope of 1.6 implies that the ethanol effect is not based on a 1:1 receptor interaction, but rather suggests a mixed effect of ethanol with more than one site of action.

Activation of BK-channels by ethanol (30 mM) was also observed in two inside-out patches with a similar range and time course of activation as found in outside-out patches (*data not shown*). Recordings from inside-out patches are difficult to obtain from GH3 cells (Armstrong & Eckert, 1987) and were therefore not further used.

To test whether the ethanol effect may be caused by osmotic effects, we performed a series of experiments replacing ethanol with ''equimolar'' concentrations of sucrose. Addition of 30 mM ethanol or sucrose to the bathing solution increased the osmolarity by 27 and 29 mosmol/l, respectively, whereas addition of 100 mM ethanol or sucrose raised the osmolarity by 119 and 101 mosmol/l, respectively. In the case of 30 mM sucrose (Fig. 4*A*) an increase of mean channel open time (15 ± 3.3%) and open probability (53 ± 15%) ($n = 6$) was observed. However, the increase of the open probability by application of 100 mM sucrose was significantly

Table. Time course of channel mean open time (*A*) and channel open probability (*B*) in the presence of external 30 mM ethanol, in the absence and presence of 1 mM ATP, 5 mM AMP-PNP in the pipette solution, and in external 300 mM ethanol.

(*A*) Mean channel open time (% increase)

Ethanol [mM]		15 sec	30 sec	60 sec
30	0 ATP	26.3 ± 9.0* (13)	−1.7 ± 11.0 (4)	7.2 ± 17.6 (4)
30	1 mM ATP	43.4 ± 12.8* (4)	12.8 ± 13.8 (4)	5.7 ± 10.5 (4)
30	5 mM AMP-PNP	−4.0 ± 7.8 (3)		
300	0 ATP	34.2 ± 3.8** (6)	9.8 ± 7.9 (6)	27.2 ± 13.6 (6)

(*B*) Channel open probablity (% increase)

30	0 ATP	78.8 ± 10.0** (13)	−20.5 ± 9.9 (4)	−0.4 ± 11.1 (4)
30	1 mM ATP	69.2 ± 19.3* (4)	5.6 ± 21.8 (4)	34.5 ± 24.3 (4)
30	5 mM AMP-PNP	20.2 ± 20.2 (3)		
300	0 ATP	317.8 ± 79.3** (6)	105.4 ± 49.9 (6)	76.2 ± 40.6 (6)

Numbers in parenthesis indicate sample size. Channel mean open time and channel open probability values are based on recordings over 12 sec starting at 15, 30 and 60 sec after onset of ethanol application. Holding potential +30 mV; *) $P < 0.05$, **) $P < 0.01$.

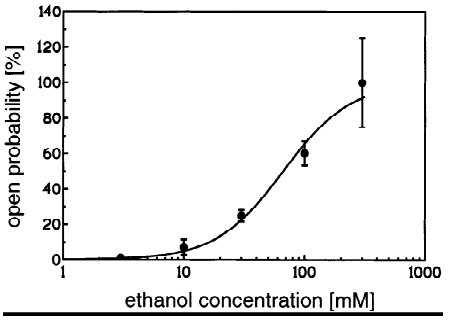

Fig. 3. Dose-response plot of the ethanol effect on channel open probability. EC_{50} = 65 mM, Hill slope = 1.6, holding potential +30 mV. Each data point represents the mean ± SEM of at least 3 experiments.

lower compared to the increase evoked by 100 mM ethanol (Fig. 4*B*). To further test if the effect of ethanol is independent from that of sucrose or not, both substances were applied simultaneously in a concentration of 30 mM each. Under these conditions an increase of mean channel open time (58 ± 14%) and open probability (130 ± 18%) was found (n = 9) (Fig. 4*A*), which closely equals the sum of both effects as expected for two substances acting via different mechanisms.

BK channels are known to be regulated by protein kinases which are closely associated with the channel protein as well as by phosphatases (Chung et al., 1991; Reinhart & Levitan, 1995). To investigate if ethanol affects the kinetics of channels by changing their phosphorylation state, we tested the ethanol effect in the presence of protein kinase- and phosphatase inhibitors. After application of 1 μM staurosporine, which is known to block protein kinase C (PKC) (Tamaoki & Nakano, 1990), channel open probability as well as mean channel open time were reduced by 46 ± 7% and 30 ± 6%, respectively (n = 12), indicating PKC to be involved in channel modulation. For the following experiments the reduced channel activity during application of the PKC blocker was taken as 100%. In staurosporine containing solution the augmenting effect of 30 mM ethanol or mean channel open time and channel open probability was prevented (Fig. 5). In contrast, addition of 30 mM sucrose in the presence of 1 μM staurosporine still significantly increased channel open probability (Fig. 5). Reduction of channel activity was also obtained using bisindolylmaleimide, another PKC blocker (Bit et al., 1993) (Fig. 5). The increase of channel mean open time in the presence of 1 μM bisindolylmaleimide and 30 mM ethanol was 11.4 ± 10% (n = 5) and in the case of open probability 24.0 ± 20% (n = 5) (compared to 26% and 79%, respectively, in the absence of blocker, *see above*), and was not significant. Again, 30 mM sucrose significantly increased the open probability by 147.4 ± 26.6% (n = 5) (*data not shown*). To further study the augmenting action of ethanol on PKC we added 500 nM PKC (19–31) pseudosubstrate, known as a selective inhibitor of PKC (House & Kemp, 1987) to the pipette solution. Under these conditions mean channel open time as well as open probability were not significantly affected by 30 mM ethanol (Fig. 5); channel mean open time was decreased by 0.5 ± 0.6%, and channel open probability was increased by 9 ± 18%, n = 5. Another three patches exhibited no channel activity at all, either under control conditions or after application of 30 mM ethanol (*data not shown*). Taken together these experiments support the notion that the ethanol effect on BK channels is not

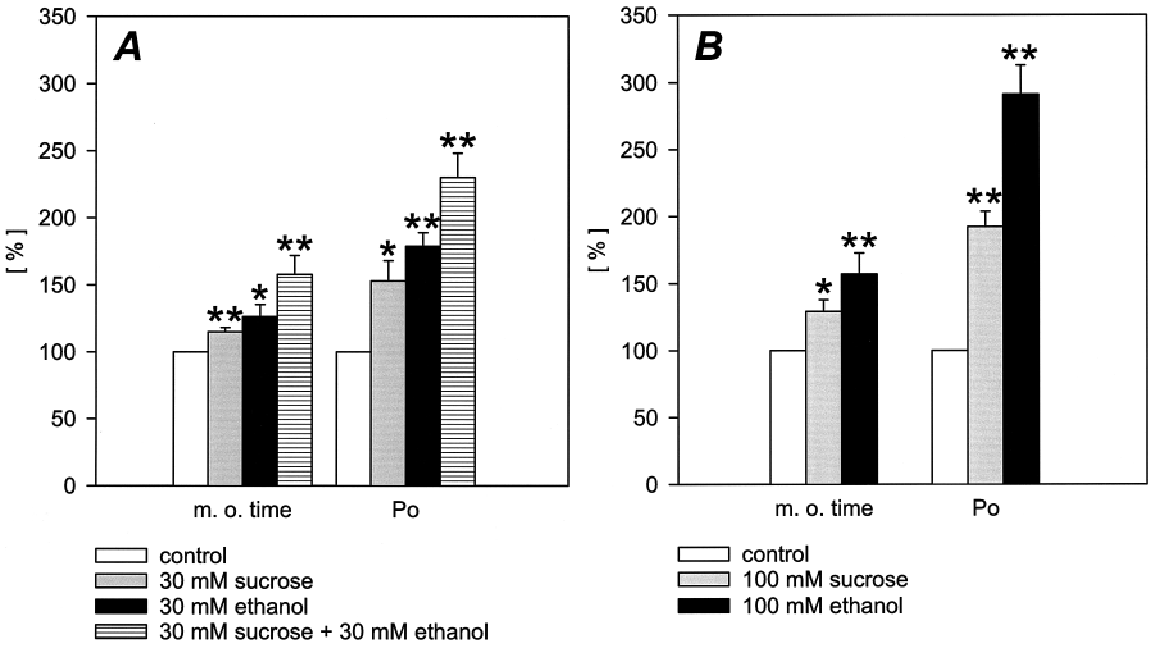

Fig. 4. Effects of ethanol vs. sucrose. (*A*) Stimulatory effect of 30 mM sucrose ($n = 6$) or 30 mM ethanol ($n = 13$), and 30 mM sucrose simultaneously ($n = 9$), and (*B*) effect of 100 mM sucrose ($n = 7$) or ethanol ($n = 8$) on mean channel open time (m. o. time) and channel open probability (P_o). The increase of open probability by 100 mM sucrose was significantly lower compared to the increase evoked by 100 mM ethanol ($P = 0.01$). Holding potential +30 mV; *) $P < 0.05$, **) $P < 0.01$.

simply an osmotic effect but is mediated via PKC stimulation. The stimulatory effect of an increased ethanol concentration to 100 mM, however, was not blocked by staurosporine or bisindolylmalemide (Fig. 6), which may be due to a competition of these blockers and ethanol or may indicate an additional site of ethanol action.

We further tested whether ethanol acts directly on PKC or indirectly via activation of phospholipase C (PLC) (Huang, 1989). After application of the PLC blocker, U-73122 (Smallridge et al., 1992), in a concentration of 1 μM, channel open probability as well as mean channel open time were found to be reduced by 84 ± 9% and 73 ± 6% ($n = 4$), which was taken as 100% for the following experiments. Under these conditions application of 30 mM ethanol significantly increased mean channel open time and open probability by 106 ± 33% and 248 ± 86% ($n = 4$), respectively, indicating that PLC is not involved in the mechanism of ethanol action.

To investigate if endogenous protein phosphatases closely associated with the channel protein (Reinhart & Levitan, 1995) are involved in channel modulation by ethanol, the protein phosphatase blockers microcystin-LR (Honkanen et al., 1990) and okadaic acid (Bialojan & Takai, 1988) were added to the pipette solution. As shown in Fig. 7, in the presence of microcystin-LR (0.25 μM) or okadaic acid (0.6 μM) channel open probability was significantly increased by ethanol (30 mM). The rise in channel open probability was even significantly higher in the presence of okadaic acid compared to the effect of ethanol in the absence of the blocker. These results suggest that (i) the blockers of phosphatases are still active in the presence of ethanol, and (ii) a block of phosphatases facilitates the stimulatory effect of ethanol.

Discussion

Ethanol applied to outside-out or inside-out patches causes a transient and concentration-dependent increase of mean open time and open probability of BK channels without affecting single-channel current amplitudes. The ethanol effect was blocked by protein kinase C antagonists and facilitated by phosphatase inhibitors. These observations are in accordance with earlier work describing an augmenting effect of ethanol on the Ca^{2+}-activated K^+ current in hippocampal CA1 neurons (Carlen et al., 1982) and snail neurons (Madsen & Edeson, 1990) as well as single BK channels in rat neurohypophyseal terminals (Dopico et al., 1996). Dopico et al. (1996) report the effect of ethanol to be independent from the presence of ATP, similar to our findings. In contrast to our results, however, they describe the ethanol effect to be sustained over a period of minutes. This might be due to a different type of BK channel in this preparation or a different regulation of the channels in neurohypophyseal terminals.

The results of our experiments where sucrose and ethanol were applied simultaneously provide strong evidence that the ethanol effect is not due to osmotic

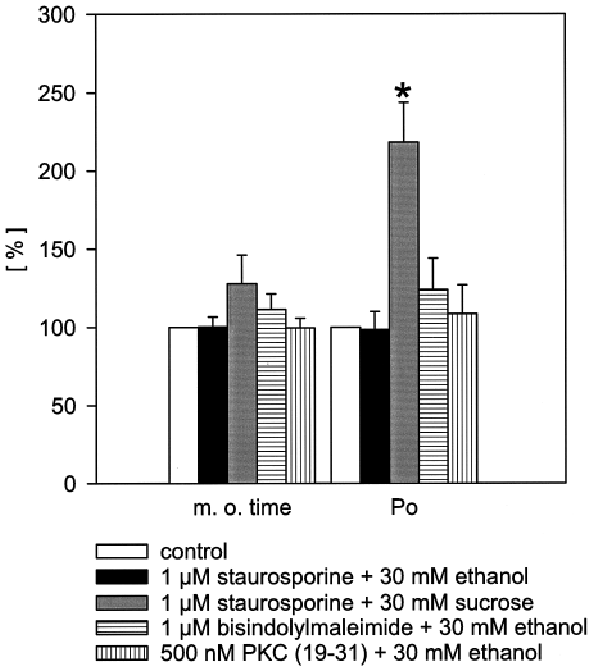

Fig. 5. Protein kinase blockers prevent the action of ethanol. In presence of the protein kinase C (PKC) blocker staurosporine (1 μM; $n = 6$), bisindolylmaleimide (1 μM, $n = 5$), and protein kinase C pseudosubstrate (19–31) (500 nM, $n = 5$) ethanol (30 mM) on mean open time of channels (m. o. time) and open probability (P_o) was ineffective. The effect of sucrose (30 mM) on the open probability, however, was not prevented by the PKC blockers ($n = 4$). Holding potential +30 mV; *) $P < 0.01$.

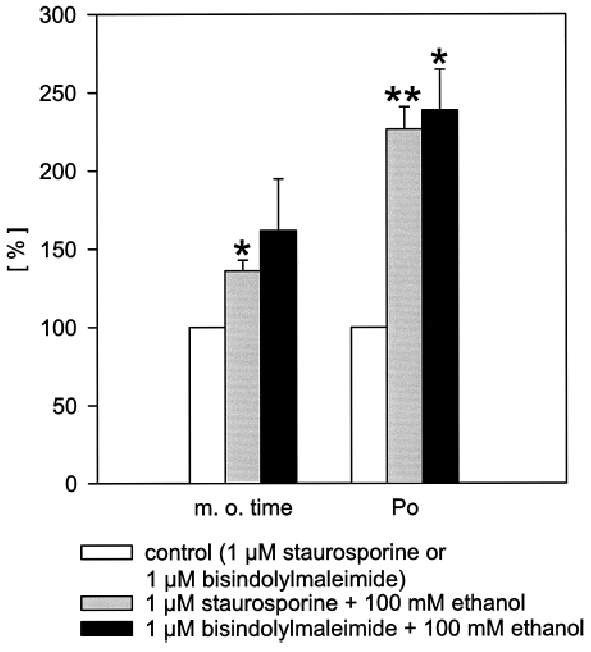

Fig. 6. Increased ethanol concentration release the effect of PKC blockers. The stimulatory effect of 100 mM ethanol is not blocked by 1 μM staurosporine ($n = 4$) or 1 μM bisindolylmaleimide ($n = 4$) and causes an increase of channel activity. Mean channel open time = m.o. time, channel open probability = P_o. Holding potential +30 mV; *) $P < 0.05$, **) $P < 0.01$.

changes since both effects were additive as expected for an independent action. Furthermore, 100 mM ethanol caused a 2-fold higher increase of channel open probability compared to an equi-osmotic solution of sucrose. In the presence of PKC inhibitors, which completely prevented the action of 30 mM ethanol, the increase of channel activity by sucrose was unaffected confirming a specific nonosmotic effect of ethanol. This might be due to the fact that ethanol, in contrast to sucrose, is able to rapidly cross the cell membrane equilibrating on both sides and thus prevents the build-up of osmotic pressure.

Channel activation was found to be independent of which side of the membrane ethanol was applied, as has been also observed by Dopico et al. (1966) for BK channels in excised patches. Furthermore, the time course of the ethanol effect in inside-out patches was similar to the one observed in outside-out patches which is consistent with the movement of ethanol through the lipid bilayer. These findings suggest that diffusion of ethanol across the membrane is not the rate-limiting step accounting for the delayed onset of the ethanol effect. The delay might rather be due to the interaction of ethanol with intracellular membrane bound targets, such as G-proteins (Rubin & Hoek, 1988; Hoffman & Tabakoff, 1990; Lahnsteiner

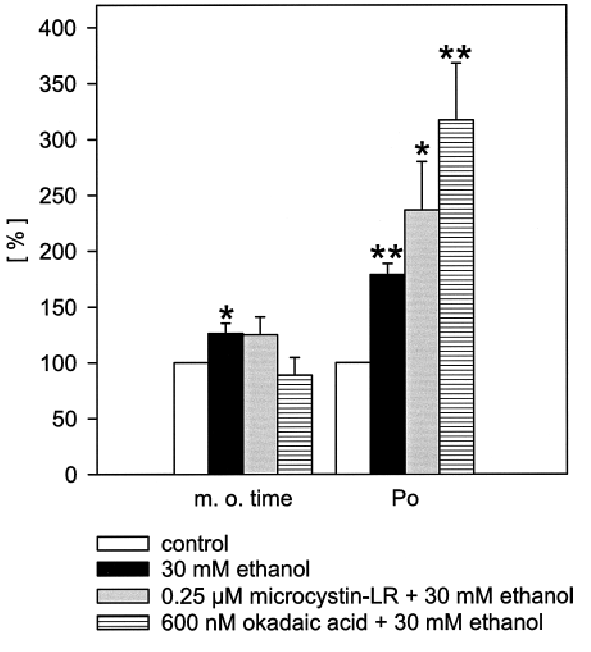

Fig. 7. Phosphatase blockers increase BK-channel activity. Effect of 30 mM ethanol on mean open time (m.o. time) and open probability (P_o) in the absence ($n = 13$) and presence of the phosphatase inhibitors microcystin-LR (0.25 μM; $n = 7$) or okadaic acid (0.6 μM; $n = 7$). Note: in the presence of okadaic acid the rise in open probability by ethanol was significantly higher than in the absence of the blocker ($P = 0.03$); Holding potential +30 mV; *) $P < 0.05$, **) $P < 0.01$.

& Hermann, 1995) or protein kinases (DePetrillo & Liou, 1993; Mironov & Hermann, 1996; Kerschbaum & Hermann, 1997), leading to an indirect modulation of BK-channel activity by channel phosphorylation.

BK channels from a variety of preparations are well known to be modulated by phosphorylation- and dephosphorylation processes including cAMP-dependent protein kinase A (PKA) (Gray et al., 1990; Carl et al., 1991; Reinhart et al., 1991), cGMP dependent protein kinase (PKG) (Alioua, Huggins & Rousseau, 1995; White, Darkow & Falvo Lang, 1995), JAK2 tyrosine kinase (Prevarskaya et al., 1995) or protein phosphatase 2A (PP-2A) (Reinhart et al., 1991). BK channels in GH4/C1 cells (Shipston & Armstrong, 1996) as well as in ovine gonadotropins (Sikdar et al., 1989) were shown to be downregulated by PKC or PKA activity. White et al. (1991) reported in GH4/C1 cells okadaic acid to inhibit BK-channel currents in intact cells. In contrast, our data from GH3 patches indicate that a block of PKC activity reduces BK-channel activity and a block of phosphatases increases the ethanol effect. It should be noted, however, that GH4/C1 and GH3 cells, although both are members of the GH-cell family, are well known to exhibit marked differences in their electrical properties (Dubinsky & Oxford, 1984) which may help to explain the different modes of BK-channel regulation by phosphorylation.

BK channels from rat brain constitute a regulatory complex consisting of the channel protein and closely associated PKC-like and protein phosphatase 1 (PP-1)-like enzymatic activities that modulate channel activity (Reinhart & Levitan, 1995). According to this work, PKC-dependent phosphorylation of BK channels leads to channel activation, whereas dephosphorylation by PP-1 phosphatase decreases channel activity which is similar to our results. There are, however, controversial reports considering direct effects of ethanol on PKC. For example, ethanol was shown to have no direct effect on the phosphorylation of specific substrate proteins ($GABA_A$ receptors) by purified PKC (Machu, Olsen & Browning, 1991). In a lipid-free assay measuring the incorporation of ^{32}P into histone III-S Slater et al. (1993) found an inhibition of purified PKC by high ethanol concentrations. On the other hand, ethanol activation of PKC resulted in a phosphorylation dependent decrease of 5-HT- and ACh-evoked Ca^{2+}-activated Cl^--channel activity expressed in *Xenopus* oocytes (Sanna, Dildy-Mayfield & Harris, 1994). Our experiments, in which the effect of low concentrations of ethanol (30 mM) on channel activity was prevented by the PKC blockers staurosporine, bisindolylmaleimide and PKC (19–31) pseudosubstrate inhibitor, indicate that PKC is activated by ethanol resulting in an increase of channel activity. These experiments also argue against a simple shift of the voltage-dependence by ethanol which is expected to be independent of PKC activity. A possible involvement of PLC in ethanol action is excluded, however, since the block of PLC was unable to prevent the ethanol effect. Neither the range of channel activation nor the time course of the ethanol effect was changed by adding 1 mM ATP to the pipette solution, suggesting remnants of ATP near the inner membrane surface after excision of the patch are sufficient for channel phosphorylation. This is further confirmed by the experiment where nonhydrolyzable AMP-PNP was added to the pipette solution, replacing ATP and thus preventing any phosphorylation dependent process possibly induced by ethanol.

The PKC blockers staurosporine and bisindolylmalemide were ineffective when higher concentrations of ethanol (100 mM) were applied. This might be due to a washout of blockers which are soluble in ethanol, or that ethanol, besides acting on PKC, has additional so far unknown target sites as suggested by a Hill slope of 1.6. Ethanol at this concentration could also cause a perturbation of the lipid environment of the channels or directly interact with hydrophobic sites in the channel protein, modulating its gating behavior as previously proposed by Dopico et al. (1996). A direct interaction with the pore-forming region can be excluded from our data, since ethanol did not change the single-channel current amplitude. The finding that the increased channel open probability by ethanol is further augmented by phosphatase inhibitors (microcystin-LR or okadaic acid) indicates that the effect of ethanol is mediated by phosphorylation of the channel protein, which is facilitated when phosphatases are blocked.

Growth hormone and prolactin producing GH3 cells as well as several other pituitary cell types (including somatotrophs, lactotrophs and gonadotrophs), show spontaneous repetitive firing of Ca^{2+}-dependent action potentials, which trigger hormone exocytosis (Stojilkovic & Catt, 1992). Ca^{2+}-activated K^+ channels contribute to spike repolarization and after-hyperpolarization and play an important role in the regulation of hormone secretion by influencing the duration and frequency of action potentials (Petersen & Maruyama, 1984; Hermann & Hartung, 1986; Marty, 1989). By these means activation of BK channels in GH3 cells by ethanol could be related to a reduced GH-release (Dees et al., 1990; Valimaki et al., 1990; Fernstorm et al., 1995). In this context it appears interesting that in GH3/B6 cells thyrotropin-releasing hormone (TRH) induces a biphasic elevation of the intracellular Ca^{2+} concentration lasting about 2 min and a reduction of the inward rectifier K^+ current (Bauer et al., 1994). Ca^{2+} elevation results in a hyperpolarization and the reduction of K^+ current in an increase of action potential frequency. Activation of BK channels by ethanol may influence either of the two phases by increasing the hyperpolarization and/or reducing the discharge activity of action potentials. Thus, even a short-term effect of ethanol on BK channels as observed in our experiments may result in perturbation of pulsatile (Van Cauter & Plat, 1996) GH-secretion.

In conclusion, our results provide evidence that ethanol increases BK-channel activity in GH3 cells via a phosphorylation dependent process and may contribute to a reduction of growth hormone secretion. At the hypophyseal level this could lead to pathological states such as depressed plasma growth hormone concentrations after ethanol ingestion.

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References

Alioua, A., Huggins, J.P., Rousseau, E. 1995. PKG-Ia phosphorylates the a-subunit and upregulates reconstituted GK_{Ca} channels from tracheal smooth muscle. *Am. J. Physiol.* **268:**L1057–L1063

Armstrong, D., Eckert, R. 1987. Voltage-activated calcium channels that must be phosphorylated to respond to membrane depolarization. *Proc. Natl. Acad. Sci.* **84:**2518–2522

Bauer, C.K., Davison, I., Kubasov, I., Schwarz, J.R., Mason, W.T. 1994. Different G proteins are involved in the biphasic response of clonal rat pituitary cells to thyrotropin-releasing hormone. *Pfluegers Arch.* **428:**17–25

Bialojan, C., Takai, A. 1988. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. *Biochem. J.* **256:**283–290

Bit, R.A., Davis, P.D., Elliott, L.H., Harris, W., Hill, C.H., Keech, E., Kumar, H., Lawton, G., Maw, A., Nixon, J.S., Vesey, D.R., Wadsworth, J., Wilkinson, S.E. 1993. Inhibitors of protein kinase C. 3. Potent and highly selective bisindolylmaleimides by conformational restriction. *J. Med. Chem.* **36:**21–29

Carl, A., Kenyon, J.L., Uemura, D., Fusetani, N., Sanders, K.M. 1991. Regulation of Ca^{2+}-activated K^+ channels by protein kinase A and phosphatase inhibitors. *Am. J. Physiol.* **261:**C387–C392

Carlen, P.L., Gurevich, N., Durand, D. 1992. Ethanol in low doses augments calcium-mediated mechanisms measured intracellularly in hippocampal neurons. *Science* **215:**306–309

Chung, S., Reinhart, P.H., Martin, B.L., Brautigan, D., Levitan, I.B. 1991. Protein kinase activity closely associated with a reconstituted calcium-activated potassium channel. *Science* **253:**560–562

Dees, W.L., Skelley, C.W., Hiney, J.K., Johnston, C.A. 1990. Actions of ethanol on hypothalamic and pituitary hormones in prepubertal female rats. *Alcohol* **7:**21–25

DePetrillo, P.B., Liou, C.S. Ethanol exposure increases total protein kinase C activity in human lymphocytes. *Alcohol Clin. Exp. Res.* **17:**351–354

Diamond, I. 1992. Alcoholism and Alcohol Abuse. *In:* Cecil Textbook of Medicine Vol. 1. J.B. Wyngaarden, L.H. Smith, J.C. and Bennett, editors. pp 44–47. Saunders, Philadelphia

Dopico, A.M., Lemos, J.R., Treistman, S.N. 1996. Ethanol increases the activity of large conductance, Ca^{2+}-activated K^+ channels in isolated neurohypophyseal terminals. *Mol. Pharmacol.* **49:**40–48

Dubinsky, J.M., Oxford, G.S. 1984. Ionic currents in two strains of rat anterior pituitary tumor cells. *J. Gen. Physiol.* **83:**309–339

Fernstorm, J.D., Parkinson, D.E., Ebaugh, A.L. 1995. Acute, oral ethanol administration suppresses episodic growth hormone secretion in the male rat. *Endocrinology* **136:**1059–1064

Gray, M.A., Greenwell, J.R., Garton, A.J., Argent, B.E. 1990. Regulation of maxi-K^+ channels on pancreatic duct cells by cyclic AMP-dependent phosphorylation. *J. Membrane Biol.* **115:**203–215

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100

Hermann, A., Hartung, K. 1983. Ca^{2+} activated K^+ conductance in molluscan neurons. *Cell Calcium* **4:**387–405

Hermann, A., Hartung, K. 1986. Pharmacological aspects of Ca-activated K-conductance in molluscan neurones. *In:* Calcium electrogenesis and neuronal firing. ed. Heinemann, U. et al. *Exp. Brain Res.* **14:**124–138

Hoffman, P.L., Tabakoff, B. 1990. Ethanol and guanine nucleotide binding proteins: a selective interaction. *FASEB J.* **4:**2612–2622

Honkanen, R.E., Zwiller, J. Moore, R.E., Daily, S.L., Khatra, B.S., Dukelow, M., Boynton, A.L. 1990. Characterization of microcystin-LR, a potent inhibitor of type 1 and type 2A protein phosphatases. *J. Biol. Chem.* **265:**19401–19404

House, C., Kemp, B.E. 1987. Protein kinase C contains a pseudosubstrate prototope in its regulatory domain. *Science* **238:**1726–1728

Huang, K.P. 1989. The mechanism of protein kinase C activation. *TINS* **12:**425–432

Jakab, M., Weiger, T.M. and Hermann, A. 1996. Ethanol activates maxi Ca^{2+}-activated K^+ channels in rat pituitary tumor cells (GH3-Cells). *In:* Göttingen Neurobiology Report. N. Elsner and H.-U. Schnitzler, editors. Vol. p. **2,** 627. Thieme Verlag, Stuttgart, New York

Kerschbaum, H.H., Hermann, A. 1997. Ethanol suppresses neuronal Ca^{2+} currents by effects on intracellular signal transduction. *Brain Res.* (*in press*)

Lahnsteiner, E., Hermann, A. 1995. Acute action of ethanol on rat hippocampal CA1 neurons: effects on intracellular signaling. *Neurosci. Lett.* **191:**153–156

Machu, T.K., Olsen, R.W., Browning, M.D. 1991. Ethanol has no effect on cAMP-dependent protein kinase-, protein kinase C-, or Ca^{2+}-calmodulin-dependent protein kinase II-stimulated phosphorylation of highly purified substrates in vitro. *Alcohol Clin. Exp. Res.* **15:**1040–1044

Madsen, B.W., Edeson, R.O. 1990. Ethanol enhancement of a calcium-activated potassium current in an identified molluscan neuron. *Brain Res.* **528:**323–326

Marty, A. 1989. The physiological role of calcium-dependent channels. *TINS* **12:**420–424

McManus, O.B. 1991. Calcium-activated potassium channels: regulation by calcium. *J. Bioenerg. Biomembr.* **23:**537–597

Mironov, S.L., Hermann, A. 1996. Ethanol actions on the mechanisms of Ca^{2+} mobilization in rat hippocampal cells are mediated by protein kinase C. *Brain Res.* **714:**27–37

Petersen, O.H., Maruyama, Y. 1984. Calcium-activated potassium channels and their role in secretion. *Nature* **307:**693–696

Prevarskaya, N.B., Skryma, R.N., Vacher, P., Daniel, N., Djiane, J., Dufy, B. 1995. Role of tyrosine phosphorylation in potassium channel activation. *J. Biol. Chem.* **270:**24292–24299

Reinhart, P.H., Chung, S., Martin, B.L., Brautigan, D.L., Levitan, I.B. 1991. Modulation of calcium-activated potassium channels from rat brain by protein kinase A and phosphatase 2A. *J. Neurosci.* **11**(6): 1627–1635

Reinhart, P.H., Levitan, I.B. 1995. Kinase and phosphatase activities intimately associated with a reconstituted calcium-dependent potassium channel. *J. Neurosci.* **15**(6):4572–4579

Rubin, R., Hoek, J.B. 1988. Alchol-induced stimulation of phospholipase C in human platelets requires G-protein activation *Biochem. J.* **254(1):**147–153

Sanna, E., Dildy-Mayfield, J.E., Harris, R.A. 1994. Ethanol inhibits the function of 5-hydroxytryptamine type 1C and muscarinic M1 pro-

tein-linked receptors in *Xenopus* oocytes expressing brain mRNA: role of protein kinase C. *Mol. Pharmacol.* **45:**1004–1012

Sikdar, S.K., McIntosh, R.P., Mason, W.T. 1989. Differential modulation of Ca^{2+}-activated K^+ channels in ovine pituitary gonadotrophs by GnRH, Ca^{2+} and cyclic AMP. *Brain Res.* **496:**113–123

Simasko, S.M. 1991. Effect of calcium on membrane potential behavior in a rat pituitary cell line (GH3). *Molecular and Cellular Endocrinology* **78:**79–86

Shipston, M.J., Armstrong, D.L. 1996. Activation of protein kinase C inhibits calcium-activated potassium channels in rat pituitary tumor cells. *J. Physiol.* **493:**665–672

Slater, S.J., Cox, K.J.A., Lombardi, J.V., Ho, C., Kelley, M.B., Rubin, E., Stubbs, C.D. 1993. Inhibition of protein kinase C by alcohols and anaesthetics. *Nature* **364:**82–84

Smallridge, R.C., Kiang, J.G., Gist, I.D., Fein, H.G., Galloway, R.J. 1992. U-73122, an aminosteroid phospholipase C antagonist, noncompetitively inhibits thyrotropin-releasing hormone effects in GH_3 rat pituitary cells. *Endocrinology* **131:**1883–1888

Stojilkovic, S.S., Catt, K.J. 1992. Calcium oscillations in anterior pituitary cells. *Endocrine Reviews* **13:**256–276

Tamaoki, T., Nakano, H. 1990. Potent and specific inhibitors of protein kinase C of microbial origin. *Biotechnology* **8:**732–735

Tashijan, A.H., Bancroft, F.C., Levine, L. 1970. Production of both prolactin and growth hormone by clonal strains of rat pituitary tumor cells. *J. Cell Biol.* **47:**61–70

Valimaki, M., Tuominen, J.A., Huhtaniemi, I., Ylikahri, R. 1990. The pulsatile secretion of gonadotropins and growth hormone, and the biological activity of luteinizing hormone in men acutely intoxicated with ethanol. *Alcohol Clin. Exp. Res.* **14:**928–931

Van Cauter, E., Plat, L. 1996. Physiology of growth hormone secretion during sleep. *J. Pediatr.* **128:**32–37

Weiger, T., Hermann, A. 1994. Polyamines block Ca^{2+}-activated K^+ channels in pituitary tumor cells (GH3). *J. Membrane Biol.* **140:**133–142

White, R.E., Darkow, D.J., Falvo Lang, J.L. 1995. Estrogen relaxes coronary arteries by opening BK_{Ca} channels through a cGMP-dependent mechanism. *Circ. Res.* **77:**936–942

White, R.E., Schonbrunn, A., Armstrong, D.L. 1991. Somatostatin stimulates Ca^{2+}-activated K^+ channels through protein dephosphorylation. *Nature* **351:**570–573