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Direct inhibition of voltage-dependent Ca^{2+} fluxes by ethanol and higher alcohols in rabbit T-tubule membranes

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Abstract

The effects of ethanol and higher alcohols on $^{45}Ca^{2+}$ fluxes, mediated by voltage-dependent Ca^{2+} channels (VDCCs), were investigated in inside-out transverse (T)-tubule membrane vesicles from rabbit skeletal muscle. $^{45}Ca^{2+}$ effluxes were induced by membrane potentials generated via establishing K^+ gradients across the vesicles, and were significantly inhibited by the inorganic Ca^{2+} channel blocker La^{3+} (1 mM) and the Ca^{2+} channel antagonist nifedipine (1–10 μM). Ethanol, in the concentration range of 100–400 mM, caused a significant suppression of depolarization-induced $^{45}Ca^{2+}$ fluxes. Ethanol also functionally modulated the effect of nifedipine (1–10 μM) and the Ca^{2+} channel agonist Bay K 8644 (1 μM) on Ca^{2+} effluxes. Pretreatment with pertussis toxin (5 μg/ml) or phorbol 12-myrstate 13-acetate (PMA, 50 nM) did not affect the ethanol inhibition of $^{45}Ca^{2+}$ fluxes. Further experiments with alcohols revealed that butanol, hexanol, octanol and decanol also significantly inhibited $^{45}Ca^{2+}$ effluxes. However, undecanol and dodecanol did not cause any significant change on $^{45}Ca^{2+}$ fluxes, indicating that the effects of alcohols on $^{45}Ca^{2+}$ effluxes exhibit a cut-off phenomenon. In radioligand binding studies, it was found that at the concentrations used in flux studies, alcohols did not alter the characteristics of the specific binding of [^{3}H]PN 200-110 to T-tubule membranes. Results indicate that ethanol directly inhibits the function of voltage-dependent Ca^{2+} channels without modulating the specific binding of Ca^{2+} channel ligands of the dihydropyridine class, and that this inhibition is independent of intracellular Ca^{2+} levels. © 2001 Published by Elsevier Science B.V.

Keywords: Ca^{2+} channel; Ethanol; Alcohol; Skeletal muscle

1. Introduction

The cellular mechanisms by which ethanol exerts its effects are as yet unclear, although a variety of receptors and ion channels have been suspected to be responsible for its inhibitory actions (for reviews, see Weight, 1992; Lovinger, 1997). There has also been considerable work suggesting that voltage-dependent Ca^{2+} channels (VDCCs) are a target site of ethanol (for a review Walter and Messing, 1999). However, there has been no report on the effect of ethanol on the function of VDCCs of skeletal muscle which, in terms of its molecular structure, is the best characterized subtype (Dunn et al., 1994). Furthermore, in earlier studies, ethanol has been shown to release Ca^{2+} from intracellular stores of several cell types, including skeletal muscle fibers, PC12 cells and oocytes (Ohnishi et al., 1984; Rabe and Weight, 1988; Wafford et al., 1989), and to interact with other second messenger systems (for a review, see Walter and Messing, 1999). The increased levels of intracellular Ca^{2+} have been shown to activate several second messenger systems, including protein kinases C, A and calmodulin (Abdel-Latif, 1986). Furthermore, VDCCs are known to be inactivated by rises in intracellular Ca^{2+} levels (Feldmeyer et al., 1993; Soldatov et al., 1998; Oz et al., 1998). Thus, in intact cell preparations, secondary effects of ethanol on intracellular Ca^{2+} homeostasis may interfere with the function of VDCCs, and need to be further investigated.

In earlier studies, we have developed a technique to measure Ca^{2+} fluxes mediated by VDCCs in purified transverse (T)-tubule membranes (Dunn, 1989; Oz et al., 1992, 1993). These membranes have been shown to form sealed, inside-out vesicles that are devoid of intracellular organelles (Dunn, 1989; Rosemblatt and Scales, 1989). Thus, Ca^{2+} flux studies in these vesicles can be used to probe the activity of VDCCs in the absence of intracellular events. In the present study, the effects of ethanol and

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higher alcohols on the function of VDCCs and on the specific binding of dihydropyridines were investigated in rabbit T-tubule membranes.

2. Materials and methods

2.1. Preparation of transverse tubule membranes

Microsomal membranes were prepared from the back and hind muscles of small (1–1.5 kg) New Zealand white rabbits, and T-tubules were isolated by sucrose gradient centrifugation as previously described (Dunn, 1989). The animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care (see Guide to the Care and Use of Experimental Animals, vol. 1 (2nd ed., 1993) and vol. 2 (1984)). T-tubule membranes were finally resuspended and equilibrated in low K^+ buffer (10 mM HEPES–Tris, pH: 7.4, 145 mM choline Cl^-, 5 mM K^+ gluconate, 0.02% NaN_3), and stored at −86°C.

2.2. $^{45}Ca^{2+}$ efflux assay

Membrane vesicles were loaded with $^{45}Ca^{2+}$ by the addition of one-half volume of isotopically diluted $^{45}CaCl_2$ solution in the same buffer, to give a final concentration of 5 mM total Ca^{2+} containing approximately 50 μCi/ml $^{45}Ca^{2+}$ (ICN, Irvine, CA, USA). After two freeze–thaw cycles to load Ca^{2+} inside the vesicles, a two-step filtration assay (Dunn, 1989) was used to investigate voltage-dependent $^{45}Ca^{2+}$ efflux. Briefly, 25 μl of loaded membranes were first diluted with 975 μl of high K^+ buffer (10 mM HEPES–Tris, pH 7.4, 120 mM K^+ gluconate, 30 mM choline Cl^-, 0.133 mM EGTA) containing 0.1 μM valinomycin and, where appropriate, the desired drug. This first dilution is designed to mimic the resting state of the cell by generating an outside negative membrane potential of −80 mV, and to reduce the extravesicular free Ca^{2+} to less than 100 nM. After 10-min incubation at room temperature, 0.9 ml was removed and applied to a GF/C filter. Excess buffer was removed by vacuum, and 1 ml of depolarizing buffer (10 mM HEPES–Tris pH 7.4, 5 mM K^+ gluconate, 145 mM choline Cl^-, 0.133 mM EGTA, 0.1 mM valinomycin) was added. Efflux was allowed to continue for 15 s prior to removal of extravesicular solution and rapid washing with two 5-ml volumes of a "stop" solution (10 mM HEPES–Tris pH 7.4, 145 mM choline Cl^-, 5 mM K^+ gluconate, 0.5 mM $LaCl_3$, 30 mM sucrose). Filters were dried, extracted with 5 ml of Hydrofluor™ (National Diagnostics, FL, USA) scintillation fluid and counted for residual entrapped $^{45}Ca^{2+}$. To ensure dissolution, alcohols with four or more carbon atoms were added to solutions from tightly sealed glass containers, vigorously shaken and sonicated for ≥30 min. The highest concentrations of undecanol and dodecanol dissolved in aqueous solution were 100 and 50 μM, respectively. Nifedipine, Bay K 8644, pertussis toxin and phorbol 12-myrstate 13-acetate (PMA) were from RBI (Natick, CT, USA). Alcohols (1-alcohols) were purchased from Aldrich (Milwaukee, WI, USA). All other chemicals were from Sigma (St. Louis, MO, USA). Dimethylsulfoxide (DMSO) at the final concentration (<0.2%), used as a solvent, did not alter $^{45}Ca^{2+}$ fluxes. Drugs and/or alcohols were incubated with $^{45}Ca^{2+}$-loaded vesicles in closed 1.5-ml microfuge tubes for 10 min on ice prior to experiments.

2.3. Binding studies

Experiments on binding of (+)-[^{3}H]PN 200-110 (Isradipine; DuPont-New England, USA) were conducted similar to our earlier studies in these membranes (Dunn, 1989). Briefly, aliquots of membranes (100 μg) were added to different concentrations of radiolabeled ligand to give a final concentration of 0.02 mg/ml T-tubule membranes in a total volume of 0.8 ml. After 60-min incubation at room temperature, 0.4-ml aliquots of each sample were filtered under vacuum through Watman GF/C filters and rapidly washed with 5 ml of ice-cold assay buffer. The filters were dried and extracted in 5 ml of Hydroflour™ (National Diagnostics) scintillation fluid before counting for ^{3}H. Triplicate 50-μl samples of the incubation mixtures were also counted directly for estimations of total binding. Nonspecific binding was estimated from parallel measurements of binding in the presence of 5 μM unlabeled nifedipine. In competition experiments, T-tubule membranes were incubated with 0.5 nM [^{3}H]PN 200-110 at a concentration of 0.02 mg/ml for 1 h, with alcohols present in the medium over a concentration range of 10 μM–800 mM.

2.4. Data analysis

All data are expressed as the arithmetic means and standard errors of the means (S.E.M.) with the number of determinations (n) indicated. The mean count per minute (cpm) was first calculated from control determinations, and then normalized to 100%. The cpm of each determination was also normalized to the mean cpm to calculate the S.E.M. for each control. Statistical evaluations were made using analysis of variance (ANOVA) method. For data analysis, calculations, nonlinear curve fittings and regression fits of the data, computer software Origin™ (Microcal Software, MA, USA) was used.

3. Results

3.1. Effect of ethanol on voltage-dependent $^{45}Ca^{2+}$ fluxes

Fig. 1A illustrates a schematic orientation of T-tubule vesicles isolated, and outlines the flux experiments carried

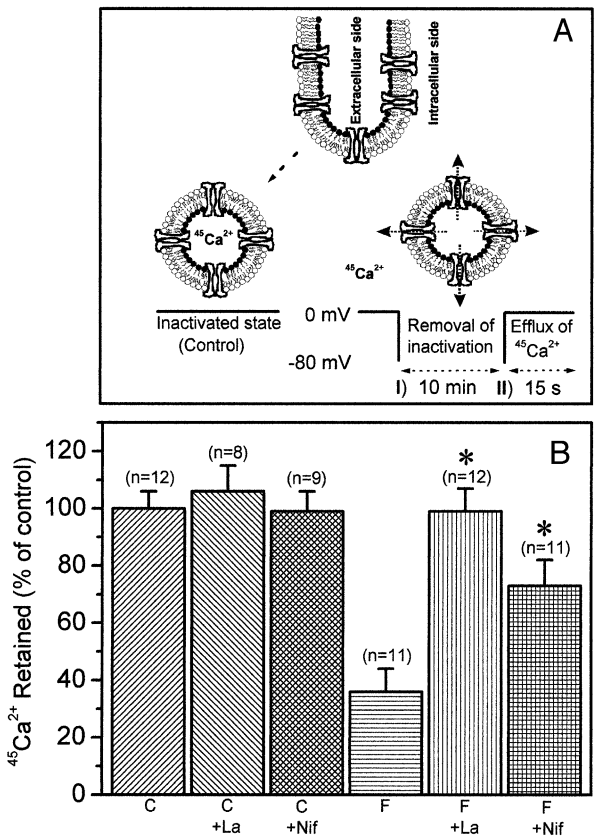

Fig. 1. Induction and pharmacological characterization of $^{45}Ca^{2+}$ flux responses through T-tubule membranes. (A) Schematic presentation of T-tubule membranes before and after isolation of inside-out vesicles (indicated with arrow). Two steps of the protocol used to induce $^{45}Ca^{2+}$ fluxes were presented with I and II, respectively. (B) Effects of membrane potential, inorganic Ca^{2+} channel blocker, La^{3+} (1 mM) and Ca^{2+} channel blocker nifedipine (10 μM) on $^{45}Ca^{2+}$ effluxes in T-tubule membrane preparations. The number of experiments (n) are presented on top of each column in all of the figures. Vertical lines on top of the columns represents the S.E.M. in all of the figures. Statistical significance at the level of $P < 0.05$ was represented by * in all figures. C, control conditions; F, flux conditions; La, lanthanum; Nif, nifedipine.

out using the two-step protocol described in Section 2. Under control conditions, there was no efflux of $^{45}Ca^{2+}$ from vesicles. Upon 15 s of depolarization, the content of $^{45}Ca^{2+}$ in the vesicles was reduced to approximately 30–40% of controls. Both nifedipine (10 μM) and La^{3+} (1 mM) significantly inhibited flux responses without affecting the amount of $^{45}Ca^{2+}$ retained in vesicles under control conditions (Fig. 1A).

Fig. 2A presents the results of experiments on the effects of ethanol on $^{45}Ca^{2+}$ efflux responses over a concentration range of 10–400 mM. At the highest concentration used (400 mM), ethanol had no effect on the $^{45}Ca^{2+}$ content of vesicles in control conditions (Fig. 2A). Ethanol in the concentration range of 10–50 mM had no significant effect on flux responses ($P > 0.05$, ANOVA, $n = 8–12$). However, in the concentration range of 100–400 mM, ethanol did cause a significant inhibition of $^{45}Ca^{2+}$ fluxes. For example, 100 mM ethanol significantly increased the amount of $^{45}Ca^{2+}$ retained in the vesicles from 35 ± 7% to 67 ± 9% of controls ($P < 0.05$, ANOVA, $n = 9–11$).

In earlier studies, it was shown that ethanol inhibition of VDCCs is mediated through pertussis toxin sensitive G-proteins and/or activation of protein kinase C pathways (Walter and Messing, 1999). For this reason, the effects of pretreatments with pertussis toxin and phorbol 12-myrstate 13-acetate (PMA), a direct activator of protein kinase C, on the ethanol inhibition of $^{45}Ca^{2+}$ fluxes were investigated. Incubating the vesicles with 5 μg/ml pertussis toxin for 1 h or 50 nM PMA for 10 min before the experiments affect neither the controls nor the ethanol-induced inhibition of flux responses (Fig. 2B). In the presence of 200 mM ethanol alone, or ethanol plus pertussis toxin, the amounts of $^{45}Ca^{2+}$ retained in vesicles were 67 ± 9%, and 64 ± 7% of the controls, respectively. There was no statistically significant difference between these values ($P > 0.05$, ANOVA, $n = 9–11$). Similarly, after pretreatment with PMA, vesicles were also tested for the effect of 10-min treatment with 200 mM ethanol on $^{45}Ca^{2+}$

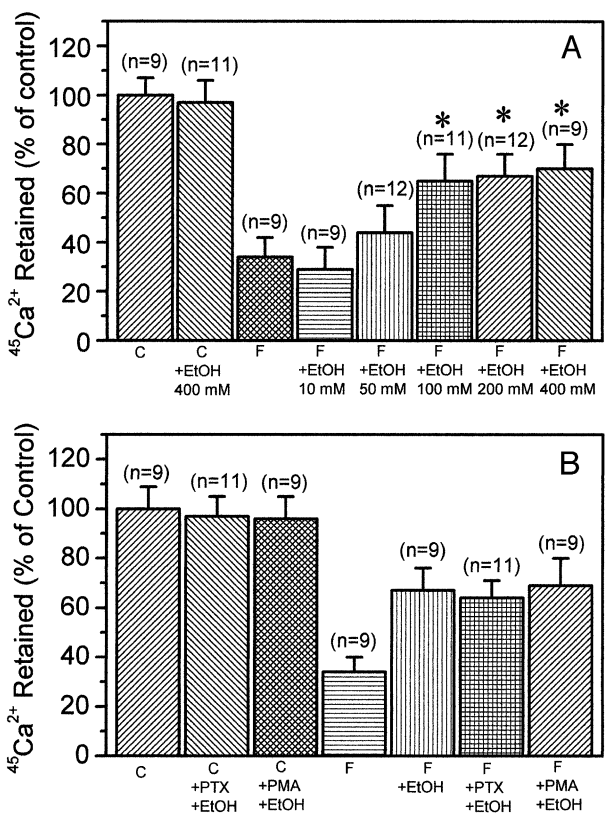

Fig. 2. Effect of ethanol on $^{45}Ca^{2+}$ fluxes through T-tubule membranes. (A) Effects of increasing concentrations of ethanol on $^{45}Ca^{2+}$ flux responses. (B) Effects of pertussis toxin and phorbol ester (PMA) on ethanol inhibition of depolarization-induced $^{45}Ca^{2+}$ effluxes. C, control conditions; F, flux conditions; EtOH, ethanol; PTX, pertussis toxin; PMA, phorbol 12-myrstate 13-acetate.

fluxes. In the presence of 200 mM ethanol alone, or ethanol plus PMA, the amount of $^{45}Ca^{2+}$ retained in vesicles was $67 \pm 9\%$ and $69 \pm 11\%$ of the controls, respectively. Difference between these values were statistically not significant ($P > 0.05$, ANOVA, $n = 9$). In presence of pertussis toxin or PMA alone, there were no significant changes in the amount of $^{45}Ca^{2+}$ retained in vesicles compared to control conditions without pertussis toxin or PMA incubations ($102 \pm 6\%$ and $97 \pm 8\%$ of controls, respectively; $n = 4$–5; data not shown).

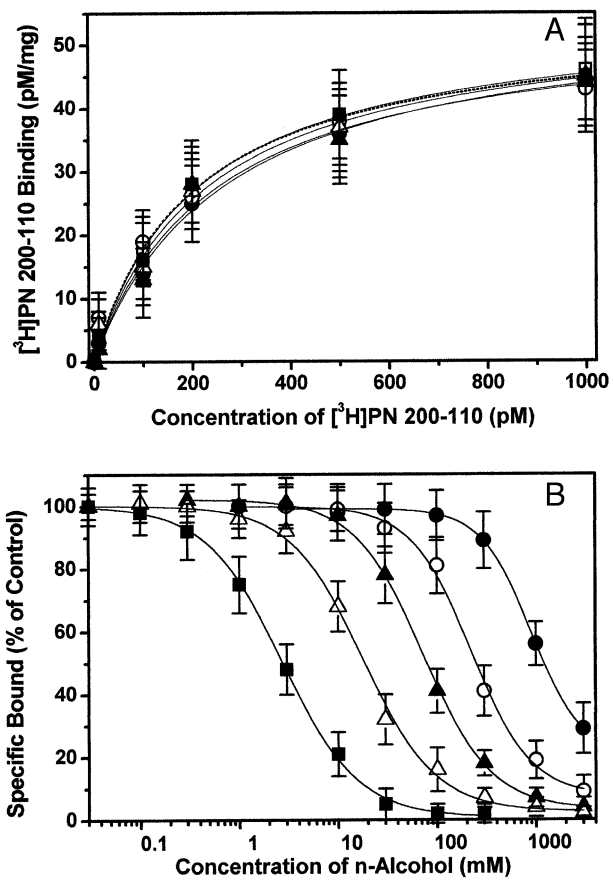

Fig. 3. The effects of ethanol and higher alcohols on the specific binding of [^{3}H]PN 200-110 to T-tubule membranes. (A) Specific binding as a function of the concentration of [^{3}H]PN 200-110. Data are presented as the arithmetic means of six experimental measurements. Data points for controls, ethanol (200 mM), butanol (20 mM), hexanol (10 mM) and decanol (0.2 mM) were represented by ●, ○, ▲, △ and ■, respectively. Solid lines were obtained with single-site binding equation using nonlinear curve fitting and represent the best fit to data points for alcohols. Fit for the control data was demonstrated with dashed line. Incubation time was 60 min at 23°C, pH 7.5. Equivalent samples were incubated with 5 μM of unlabeled nifedipine in order to determine non-specific binding. (B) The effect of increasing concentrations of alcohols on the specific binding of [^{3}H]PN 200-110 to T-tubule membranes. Data are expressed as percentage of control. The IC_{50} values were obtained from nonlinear regression fits of the data points and are reported in the text. T-tubule membranes were incubated with 0.5 nM [^{3}H]PN 200-110 at a concentration of 0.02 mg/ml for 1 h, with increasing concentrations of alcohols in the medium. Bound and free [^{3}H]PN 200-110 were separated by filtration. Symbols are the means of four to five experiments. Data points for ethanol, butanol, hexanol octanol and decanol were represented by ●, ○, ▲, △ and ■, respectively.

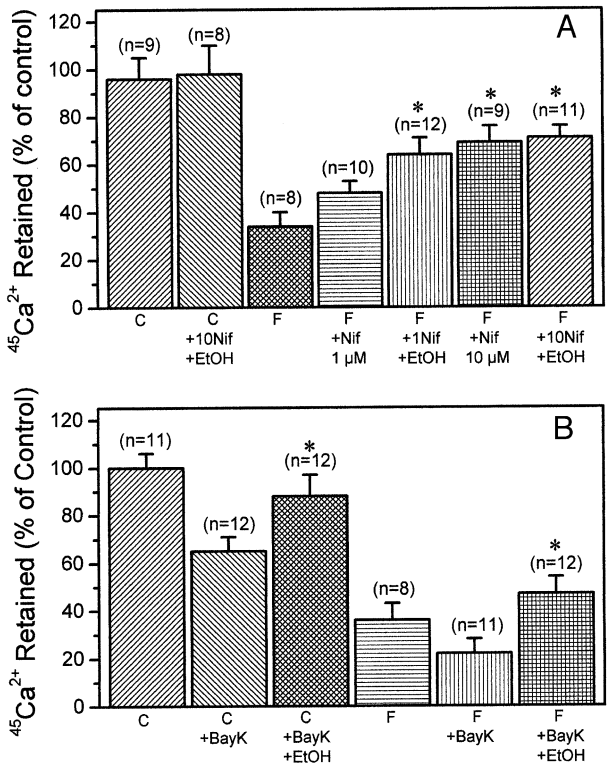

Fig. 4. Functional interaction between the effects of dihydropyridine-class VDCC modulators and ethanol on depolarization-induced $^{45}Ca^{2+}$ effluxes in T-tubule vesicles. (A) Effects of incubating T-tubule membranes with 1 or 10 μM of nifedipine, in the presence and absence of 200 mM ethanol, on $^{45}Ca^{2+}$ effluxes. (B) Effects of incubating T-tubule membranes with 1 μM Bay K 8644, in the presence and absence of 200 mM ethanol, on $^{45}Ca^{2+}$ effluxes. C, control conditions; F, flux conditions; Nif, nifedipine; BayK, Bay K 8644; EtOH, ethanol.

3.2. Effect of ethanol on the binding of [^{3}H]PN 200-110

Fig. 3A illustrates the amount of the specific binding of [^{3}H]PN 200-110 to purified T-tubule vesicles, in the absence and presence of ethanol and higher alcohols at the concentrations which caused a significant inhibition of $^{45}Ca^{2+}$ fluxes. Alcohols, including ethanol, did not cause an apparent change on the characteristics of the specific binding of [^{3}H]PN 200-110 ($P > 0.05$, ANOVA, $n = 5$–6). The apparent affinity (K_D) of the receptor for [^{3}H]PN 200-110 were 0.21, 0.23, 0.24, 0.23, 0.20 and 0.21 nM for controls, ethanol (200 mM), butanol (20 mM), hexanol (10 mM), octanol (2 mM) and decanol (0.2 mM), respectively. Values for maximum bindings (B_{Max}) of [^{3}H]PN 200-110 were 52.1, 53.7, 52.3, 52.8, 49.2 and 52.9 pM/mg of protein for controls, ethanol, butanol, hexanol, octanol and decanol, respectively.

At various concentration ranges, both ethanol and higher alcohols were also investigated for their ability to displace the specific binding of [^{3}H]PN 200-110 from rabbit T-tub-

ule membranes (Fig. 3B). In the concentration range used, alcohols inhibited the specific binding of [^{3}H]PN 200-110. The IC_{50} values for ethanol, butanol, hexanol, octanol and decanol were 897.2, 221.9, 71.6, 17.7 and 2.8 mM, respectively. At the highest concentrations tested, higher alcohols including undecanol (100 μM) and dodecanol (50 μM) caused 19.7 ± 6% ($n = 4$) and 16.3 ± 5% ($n = 5$) inhibition of specific binding, respectively. However, they were not further studied due to their incomplete solubilities in aqueous solutions.

3.3. Further investigations on the mechanism of ethanol inhibition

Functional interactions between ethanol and dihydropyridine-class Ca^{2+} channel antagonist nifedipine and agonist Bay K 8644 on $^{45}Ca^{2+}$ effluxes were also studied. In these experiments, T-tubule vesicles were incubated with 1–10 μM nifedipine or 1 μM Bay K 8644 in presence and absence of 200 mM ethanol. Results of these experiments were demonstrated in Fig. 4A. Ethanol significantly increased the extent of inhibition by 1 μM nifedipine. In vesicles treated with 1 μM nifedipine, $^{45}Ca^{2+}$ contents were 64 ± 7% and 48 ± 5% of controls, in the presence and absence of 200 mM ethanol, respectively ($P < 0.05$, ANOVA, $n = 10$–12). On the other hand, in vesicles treated with 10 μM nifedipine, 200 mM ethanol did not affect the inhibition of flux responses by nifedipine (Fig. 4A; $P > 0.05$, ANOVA, $n = 9$–11).

The results of experiments investigating the functional interaction between 1 μM Bay K 8644 and 200 mM

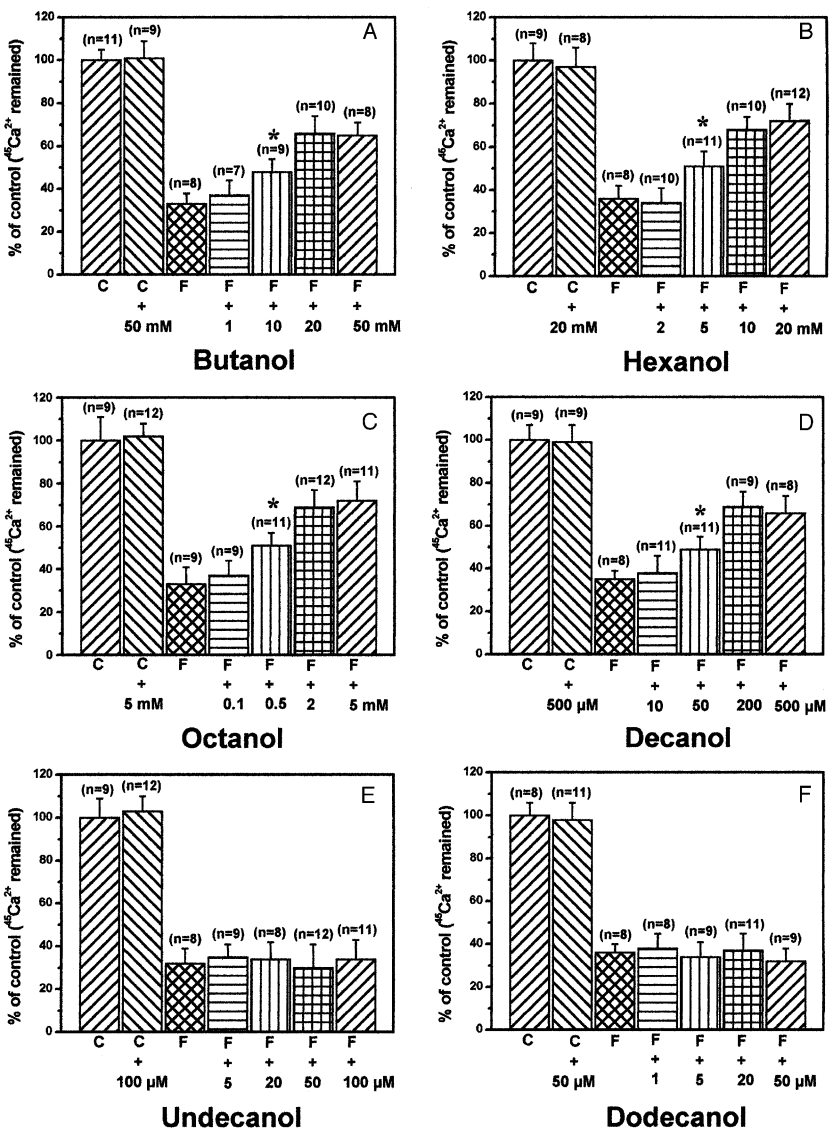

Fig. 5. The effects of increasing the carbon chain-length of alcohols on $^{45}Ca^{2+}$ effluxes through T-tubule membranes. The histogram panels demonstrates the effect of increasing concentrations of butanol (A), hexanol (B), octanol (C), decanol (D), undecanol (E) and dodecanol (F) on $^{45}Ca^{2+}$ content of vesicles. On the bottom of the columns in each panel, C and F represent control and flux conditions, respectively. The concentrations of alcohols added to control or efflux conditions were also presented at the bottom of the each column on the panels. At the level of $P < 0.05$, "threshold" concentration of alcohols caused a statistically significant inhibition of $^{45}Ca^{2+}$ fluxes were represented by *.

ethanol were presented in Fig. 4B. Under control conditions, Bay K 8644 induced effluxes (65 ± 6% of controls) significantly inhibited by 200 mM ethanol (87 ± 9% of controls, $P < 0.05$, ANOVA, $n = 12$). The effect of Bay K 8644 on flux responses was also significantly suppressed by 10-min coincubation of vesicles with ethanol and Bay K 8644. In the presence of ethanol, the amount of $^{45}Ca^{2+}$ retained in Bay K 8644-treated vesicles was significantly increased from 22 ± 4% to 46 ± 7% of controls ($P < 0.05$, ANOVA, $n = 11–12$).

3.4. Effects of different chain-length alcohols on $^{45}Ca^{2+}$ fluxes

These sets of experiments were designed to investigate whether a cut-off phenomenon can be observed on the inhibition of $^{45}Ca^{2+}$ fluxes by alcohols. Similar to experiments with ethanol, effects of 10-min incubation of vesicles with alcohols of different carbon chain-lengths were tested under control and depolarizing conditions. The results of these experiments were presented in Fig. 5A–F. At the highest concentrations used, alcohols, tested up to dodecanol (carbon chain-length of 12), did not cause an appreciable effect under control conditions. Initial experiments revealed that the concentrations of alcohols required for their effect drastically decreased with increasing the carbon chain-length of the alcohol. In panels A–F of Fig. 5, the threshold concentrations that caused statistically significant inhibition of $^{45}Ca^{2+}$ fluxes were indicated on top of the bars. On each panel, only the subthreshold, threshold and suprathreshold concentrations of alcohols were presented (Fig. 5A–F). Up to decanol (carbon chain-length of 10), all of the alcohols tested effectively inhibited $^{45}Ca^{2+}$ fluxes (Fig. 5A–D). On the other hand, beginning after decanol, there was an abrupt loss of the effect of alcohols on $^{45}Ca^{2+}$ fluxes. Undecanol and dodecanol (tested at the maximum concentrations possible to dissolve in aqueous environment) did not have any significant effect on $^{45}Ca^{2+}$ fluxes (Fig. 5E,F).

4. Discussion

The major finding of this investigation is that ethanol directly inhibits the function of VDCCs in T-tubule membranes, and this effect is independent of intracellular Ca^{2+} levels. Although in earlier studies, suppression of VDCCs by ethanol and other alcohols has been demonstrated in cardiac and smooth muscle preparations (Hawtorn et al., 1992; Habuchi et al., 1995), to our knowledge, this is the first report indicating that ethanol directly inhibits the function of L-type VDCC in isolated muscle membranes.

In T-tubule membranes, the abundant presence of specific binding sites for dihydropyridines (Fosset et al., 1983; Dunn, 1989), functional Ca^{2+} channels corresponding to these binding sites (Flockerzi et al., 1986) and stereospecific modulation of $^{45}Ca^{2+}$ flux responses by dihydropyridines and other classes of Ca^{2+} channel antagonists (Dunn, 1989; Oz et al., 1992, 1993) have been demonstrated. Since changes in the lipid compositions of bilayer membranes have been reported to affect the functional properties of VDCCs (Coronado, 1987), T-tubule vesicles have the additional advantage of containing VDCCs in their native membranes. Using fluorescent voltage sensitive dyes, voltage-changes predicted by the Nernst equation have also been recorded in T-tubule vesicles depolarized by changes in the extravesicular concentration of K^+ (Dunn, 1989). These findings indicate that $^{45}Ca^{2+}$ fluxes in these vesicles are mediated through VDCCs. T-tubule vesicles used in the present study are in an inside-out orientation. They are also devoid of intracellular organelles and plasmalemmal Ca^{2+} pump activity (Dunn, 1989; Rosemblatt and Scales, 1989). Thus, it is unlikely that the observed inhibitory effects of ethanol or other alcohols on $^{45}Ca^{2+}$ fluxes through inside-out oriented vesicles involve changes in intracellular Ca^{2+}.

Similar to earlier studies on cardiac and smooth muscle (Hawtorn et al., 1992; Habuchi et al., 1995), ethanol at 100 mM and higher concentrations significantly inhibited the flux responses (Fig. 1B). On the other hand, our attempts to obtain more gradual flux responses by decreasing the depolarization time to 5 s and/or using partial depolarizing conditions were not successful. Hence, it was not possible to obtain further information on the quantitative aspects of the concentration–response relation for the effect of ethanol and other alcohols.

It is likely that a certain portion of the total $^{45}Ca^{2+}$ fluxes, but not all, is carried through L-type VDCCs in T-tubule vesicles. Firstly, there may be some nonspecific binding of $^{45}Ca^{2+}$, presumably to divalent cation binding sites on lipid bilayers. The finding that even after depolarization of vesicles pretreated with Bay K 8644, 15–25% of the total $^{45}Ca^{2+}$ remains in vesicles, is in agreement with the presence of such binding sites (Figs. 1A and 4B). It is also likely that a differential contribution of this bound Ca^{2+} to each batch of membrane vesicles may cause some variations among different batches of membranes. Secondly, the presence of T-type Ca^{2+} channels in T-tubules of amphibian and mammalian skeletal muscles has been reported (Garcia and Stefani, 1987; Dirksen and Beam, 1995). Although under the prolonged (15 s) depolarizations of vesicles used in this study, T-type channels should largely be inactivated, some $^{45}Ca^{2+}$ flux through these channels during the early phases of depolarization may interfere with our results. In fact, nifedipine (10 μM), which completely abolish the Ca^{2+} currents mediated by L-type VDCCs without affecting T-type VDCCs in mammalian skeletal muscle fibers (Lamb and Walsh, 1987; Dirksen and Beam, 1995), did not block the $^{45}Ca^{2+}$ fluxes completely. The remaining 30–35% of total flux response resistant to inhibition by ethanol or nifedipine may reflect

the contribution of T-type channels to total fluxes. In line with this conclusion, almost 100% inhibition of flux responses by La^{3+} (Fig 1A), which effectively blocks both L- and T-type VDCCs (Mlinar and Enyeart, 1993; Dirksen and Beam, 1995), may also imply the contribution of T-type VDCCs to total $^{45}Ca^{2+}$ fluxes.

The inhibitory effect of nifedipine (1 μM) on $^{45}Ca^{2+}$ fluxes was significantly enhanced in the presence of ethanol. In addition, the effect of ethanol was masked at saturating concentrations of nifedipine (10 μM). These findings may suggest that the inhibitory effects of ethanol and nifedipine are mediated through a common ionic pathway. Furthermore, ethanol reversed the agonist effect of Bay K 8644 on $^{45}Ca^{2+}$ fluxes, suggesting that ethanol is also functionally modulating the effects of dihydropyridines on L-type VDCCs.

Results indicate that although alcohols, at high concentrations, displaced the specific binding of [^{3}H]PN 200-110, this effect is not likely to account for their inhibitory effects on $^{45}Ca^{2+}$ fluxes. In earlier studies, it was also found that ethanol, at concentrations which inhibited $^{45}Ca^{2+}$ fluxes, did not affect dihydropyridine binding sites in brain membranes (Greenberg and Cooper, 1984; Canda et al., 1995). Similarly, in smooth muscle membranes, K^{+}-induced contractions were more sensitive to the inhibitory effects of ethanol and higher alcohols than the displacement of dihydropyridine ligand binding (Hawtorn et al., 1992). These findings, in line with our results, suggest that the displacement of dihydropyridine ligand binding is not the mechanism of action of ethanol on $^{45}Ca^{2+}$ fluxes. The results also suggest that the effect of ethanol on skeletal muscle VDCCs is not mediated by pertussis toxin-sensitive G-proteins ($G_{i/o}$) and/or by the direct activation of protein kinase C.

Recent findings strongly suggest that alcohols and general anesthetics exert their effects by acting directly on the proteins, rather than disturbing the structure of lipid-bilayer membranes (for reviews, see Franks and Lieb, 1994; Peoples et al., 1996). An important piece of evidence supporting the protein hypothesis is the loss of the biological effects of alcohols when their carbon chains exceed a certain length. This property was originally described in firefly luciferase (Franks and Lieb, 1985) and observed on ligand and voltage-gated ion channels (for reviews, see Peoples et al., 1996; Lovinger, 1997). It is known as the "cut-off" phenomenon (Franks and Lieb, 1985, 1994). In this study, we have also investigated whether the effect of alcohols reaches a cut-off level by increasing the carbon chain-lengths of alcohols. Our findings revealed a cut-off phenomenon at undecanol. This further supports the hypothesis that the action site of ethanol is on the ion channel. This is the first report describing this phenomenon on VDCCs of skeletal muscle. In an earlier study on the inhibition of Na^{+} action potentials by primary alkanols, cut-off was found at the level of dodecanol (Requena et al., 1985). Another study on neuronal Na^{+} channels revealed a cut-off at the level of undecanol (Rodriguez et al., 1988).

It is likely that the binding site of alcohols is a hydrophobic pocket on the channel-protein. Since undecanol and dodecanol were ineffective on $^{45}Ca^{2+}$ fluxes, the pocket on VDCCs of skeletal muscle can accommodate alcohols with up to 10 carbons in length. Considering the structural homologies between the subunits of Na^{+} and Ca^{2+} channels (Catterall, 1988; Dunn et al., 1994), hydrophobic pockets with similar sizes may be a site of alcohol action on these proteins.

Recent studies in single mouse skeletal muscle fibers (Cofan et al., 2000) and in cultured human myotubes (Nicolas et al., 1998) report that acute application of ethanol significantly decreases Ca^{2+} transients. These studies measured Ca^{2+} fluxes indirectly by the Mn^{2+} quenching of fura-2 at the cellular level and, in agreement with our results, suggested that the inhibitory effect of ethanol is primarily due to the inhibition of sarcolemmal Ca^{2+} influx via nitrendipine-sensitive VDCCs. In amphibian muscles, the role of the entrance of extracellular Ca^{2+} through VDCCs during maintained mechanical responses, such as tetanic contractions has been suggested in earlier studies (Oz and Frank, 1991). It is likely that ethanol inhibition of VDCCs may contribute to suppression of tetanic contractions by ethanol in amphibian (Oz and Frank, 1995) and mammalian skeletal muscles (Pagala et al., 1995).

In conclusion, our results suggest that ethanol directly inhibits the function of VDCCs in purified T-tubule membrane vesicles. It appears that the inhibition of channel function is not related to the DHP binding site on the channel, and that it is not mediated by the activation of pertussis toxin sensitive G-proteins and/or a protein kinase C pathway.

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