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Effects of novel antituberculosis agents on OmpF channel activity

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ABSTRACT

Nanopore forming proteins spanning the outer membrane mediate in the diffusion of hydrophilic chemicals through the hydrophobic bacterial cell wall. In this study, the effects of two novel anti-TB derivatives, ethyl α-[5-(5-nitro-2-thienyl)-1,3,4-thiadiazole-2-ylthio] acetates and propyl α-[5-(5-nitro-2-thienyl)-1,3,4-thiadiazole-2-ylthio] acetates, on OmpF channel reconstituted in artificial bilayers were evaluated by voltage clamp technique. Surprisingly, ethyl derivative (MIC ⩾ 6.75 μg/ml) showed no effects on OmpF channel activity but the propyl derivative (MIC = 0.39 μg/ml) reduced the channel conductance considerably and changed the gating pattern of the channel. The findings obtained here at molecular level, might shed light on better understanding of the actual mechanism(s) by which the novel anti-TB agents permeate through the cell wall of the *Mycobacterium tuberculosis*.

OmpF is the most studied membrane porin channel whose various characteristics including genetic diversity [1–3], expression requirements [4,5], molecular structure [6–8], selectivity [9,10], gating characteristics and voltage sensitivity [11–13], pH sensitivity [14] and blocking agents [15–17] have been thoroughly investigated. Furthermore, the mechanism(s) by which the channel responds to the membrane potential difference [18–20] have been studied experimentally. However, due to limitation in the sensitivity and speed of data acquisition, theoretical approach and molecular dynamics simulation investigations [21–23] as well as mathematical analysis of the channel activities [24,25] have also applied to understand the channel dynamics at molecular level.

Conductance of OmpF porin channel is dependent on the concentration of the current carrying ions present in the medium as well as other hydrophilic and hydrophobic molecules (and/or blockers). The conductance of fully open monomer (full state) reported as 1400 pS in 1 M KCl at neutral pH, though, lower conductances with size of one half or even less are also produced that correspond to sub-states and small channels, respectively [12].

OmpF porin channel is voltage sensitive so that its open probability, P_o, decreases as potential difference (pd) across the channel increases. The channel is fully open at voltages less than 60 mV and its monomers close one by one as pd exceeds ±160 mV. At medium voltages ionic current fluctuates frequently between minimum (closed) and maximum (open) level [12,26]. The higher the voltage, the lower is the ratio of mean closed time to mean open time. Furthermore, there are occasions when the channel gates at much higher frequencies, so-called fast flickering, when channel equally spent in open and closed states [12]. In most cases the channel shows a symmetrical behavior at different polarities of the applied pd. One should note that voltage sensitivity of the channel is pH dependent and the mentioned values are obtained at neutral pH. The lower the pH, the higher is the voltage sensitivity of the channel, resulting in smaller P_o at lower pH [14].

Translocations of β-lactam antibiotics through OmpF porin channels have also been studied [24,25]. It has been shown that moving molecules through the channel lumen cause transient complete/partial obstruction of ion flow that is monitored by means of current caused by the applied pd. The length of obstruction time analyzed by single channel recording claimed to be in the order of microsecond [27].

According to the recent reports published by WHO, tuberculosis is actively spreading [28] and its treatment is rather difficult and time consuming process [29]. Of the most recent synthesized drugs proved to be effective on *Mycobacterium tuberculosis in vitro* are cyclic compounds, 1,3,4-thiadiazole derivatives, whose MIC, extent of growth inhibition, rate of activity and etc were reported [30]. There are several biological and antibacterial effects reported for the thiadiazole ring [31].

In this study, due to the importance of antibacterial drug uptake by the bacteria, and the corresponding mechanism(s), we have studied the effects of anti-tuberculosis thiadiazole derivatives on the well characterized OmpF channel reconstituted in artificial membranes. We have shown that amongst the thiadiazole derivatives, propyl ester and not ethyl ester was capable of regulating the channel molecular activities, and keeping it in a stable closed state.

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Materials and methods

Soybean (Type IIS) obtained from Sigma, octyl-polyoxyethylene (Octyl-POE), from Bachem & Feinchemikalien, and 2-amino-5-(5-nitro-2-thienyl) 1,3,4,-thiadiazole from Laboratory of Organic Chemistry, Faculty of Pharmacy, Tehran University of Medical Sciences. OmpF wild type porin was extracted and purified from *Escherichia coli* K12 by H. Mobasheri. The remaining chemicals used were obtained from Merck.

Thiadiazole derivatives, ethyl acetate and propyl acetate were synthesized and purified according to the method published by Foroumadi et al. [30]. Briefly, deazotation of 2-amino-5-(5-nitro-2-thienyl)-1,3,4-thiadiadzole (**1a**) in the presence of copper powder in hydrochloric acid produced 2-chloro-5-(5-nitro-2-thienyl)-1,3,4-thiadiazole (**2a**). The reaction between (**2a**) with thiourea in refluxing ethanol produced 2-mercapto-5-(5-nitro-2-thienyl)-1,3,4-thiadiazole defined as (**3a**). Treatment of (**3a**) with ethyl or propyl chloro acetates (**4a**–**b**) produced alkyl α-[5-(5-nitro-2-thienyl)-1,3,4-thiadiazole-2-ylthio] acetates (**5a**–**b**) (Fig. 1). The structure of synthesized products, were further evaluated by means of ^{1}H NMR spectra, recorded on a Bruker AC-300 spectrometer. The mass spectra of samples were also run on a Finigan TSQ-70 Spectrometer at 70 eV.

Artificial planar bilayers were formed based on Montal and Mueller technique [32], and its characteristics evaluated by applying a square wave of 120 Hz, 2Vpp. Having worked out the impermeability and stability of the membrane at pd's as high as ±180 mV, protein in Octyl-POE (1%), KCl 1 M, was introduced to the *cis* compartment making a final concentration of about 50 nM, in presence of less than 0.1% Octyl-POE. The working buffer in both *cis* and *trans* compartments consisted of $CaCl_2$ 10 mM, KCl 1 M, HEPES 10 mM at pH 7.4. The current signals recorded by PAT 6 software (J. Dempster, Stratclyde University), were filtered by means of two low pass filters at 9 KHz, amplified as required (5–500 times) and digitized by CED Cambridge Instruments. The analyses of current signals were carried out statistically and also practically using PAT6 and PAT7.

Results

The possibility of translocation as well as the interaction of the thiadiazole derivatives with reconstituted OmpF porin channel was studied at molecular level. The channel activities monitored at single channel level, focusing on its various characteristics including conductance, voltage sensitivity, gating properties, etc. The Channel showed a monomer conductance of 1283 ± 20 pS at positive potential and 1246 ± 15 pS at negative potential on the *cis* side. The voltage sensitivity of the channel was consistent with the results reported by other groups [12,13], closing at about ±150 mV. Compound (**5b**) was added to the *cis* compartment with a final concentration of 90 μM, while monitoring and recording channel activity at 60 mV, where the channels were mainly open. Significant changes in gating pattern of the channel in negative potential were monitored after 10 min (Fig. 2A). The effect of the added chemical on channel gating was not symmetrical at different polarities, i.e., fast gating was monitored at negative polarity.

The detailed analysis of channel activity at higher resolution showed fast flickering (gating) at high frequency (Fig. 2A) as well as mini-channels next to the main states. These channels were the last channels that closed when the pd is increased.

Following the addition of compound (**5b**), the conductance of the channel decreased and tended to force the channel to close at low pd's. The resulting conductances, 2704 ± 43 pS at positive potential and 2628 ± 50 pS at negative potential, seems to correspond to the conductance of dimmers. Furthermore, new stable substates were formed in the presence of (**5b**) when high pd's irrespective of the polarity were applied. However, at lower pd's substates were formed in large numbers only when negative pd's were applied to the *cis* side.

Voltage sensitivity of OmpF channel in the presence of (**5b**) increased, so that the channel tend to close mainly at pd's as low as ±60 mV. The response of the channel to different pd's in the presence and absence of (**5b**) is shown in Fig. 2B.

In order to further analyze the involvement of compound (**5b**) in the formation of these substates, the substates and main states distribution of the channel conductance recorded in three independent experiments were analyzed (Fig. 2C). Fully open trimer (4350 ± 162 pS) was not monitored anymore and the channel showed a conductance of 2790 ± 313 pS that could correspond to a dimer with a conductance of smaller than the expected value of 3063 ± 274 pS. The significance of the value was worked out and the *P* value of <0.05 produced by the analysis of 132 conductance values recorded from channels in the absence of (**5b**) and

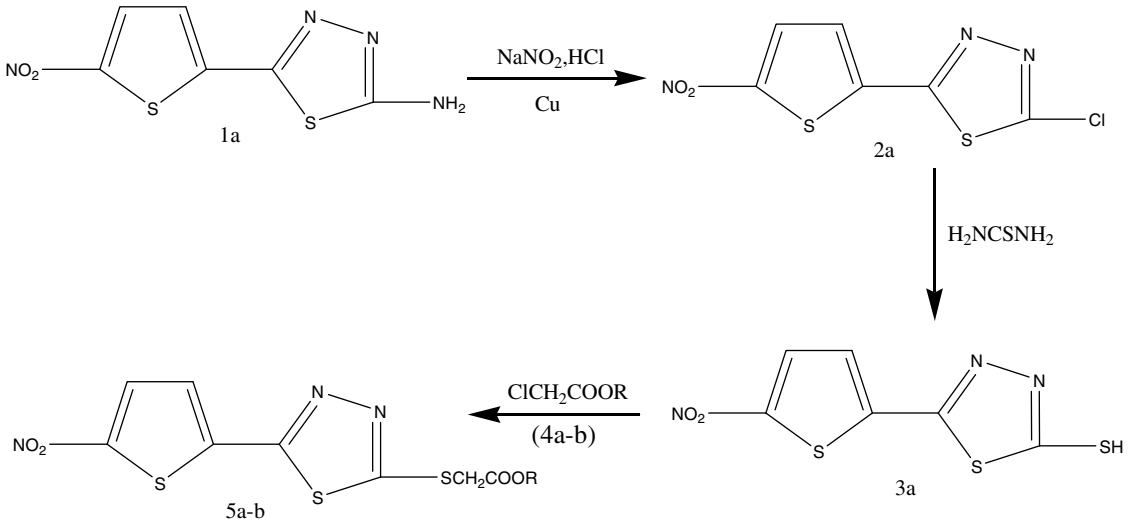

Fig. 1. Synthesis of alkyl α-[5-(5-nitro-2-thienyl)-1,3,4-thiadiazole-2-ylthio] acetates defined as (**5a**–**b**).

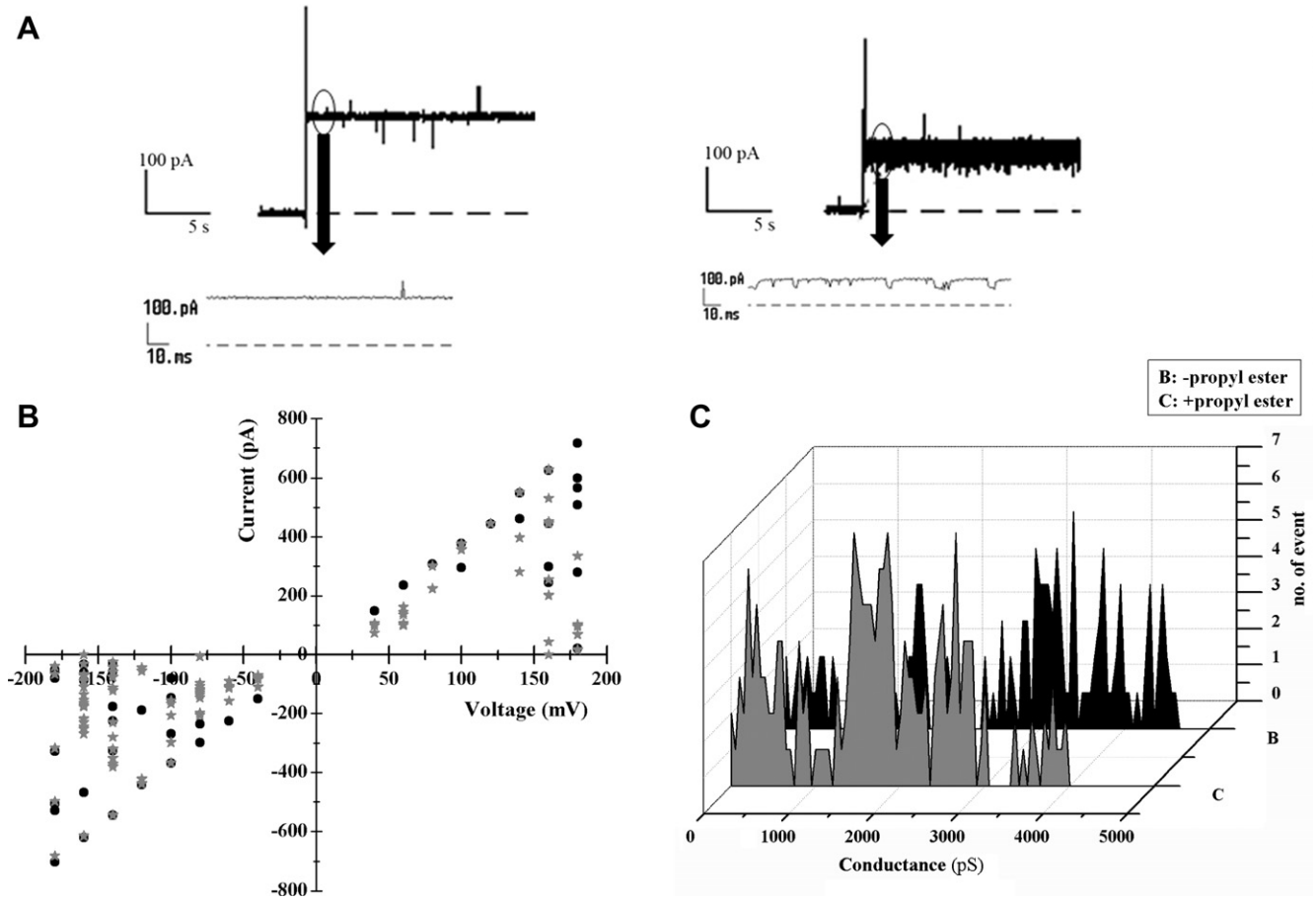

Fig. 2. (A) OmpF trimer gating at −60 mV, in the absence (left) and presence (right) of propyl ester derivative added to the *cis* side. The positive current is produced when negative polarity of pd is applied to the cis side. (B) The response of OmpF to different applied pd's in the presence (stars) and absence (circles) of propyl ester derivative added to the *cis* side. (C) Conductance distributions of the activities of about 331 channel conductance values, recorded in three different membranes.

199 conductance values in its presence, confirming the accuracy of the dimer conductance reported.

The channel mean open time varied in response to the applied pd. Though, short time closures recorded at low pd's (±40 mV), long ones, i.e., complete closure took place only when the pd reached +80 mV (Fig. 3B). The channels did not lock at closed state, and reopened as soon as the pd switched to zero. Furthermore, gating frequency of the channel was quite high at low positive potential differences and declined to very low level as pd increased.

In order to verify OmpF channel activity due to the direct effect of compound (**5b**) on the lumen compared with that of through the channel's lipid medium, following the recording of the channel activity in the presence of (**5b**), the *cis* compartment was perfused to eliminate the induced gradient concentration effect of (**5b**) on the lumen (Fig. 3A). The perfusion experiment did show that once the (**5b**) was introduced, the resulting fast gating continued, and its removal did not change the original gating pattern.

Reintroduction of compound (**5a**) to the *cis* compartment with the same concentration (90 μM) did not change the channel activity pattern and the conductance size of the channel (Fig. 4A). Furthermore, the frequency of the channel gating solely produced at negative potential, remained constant in the presence and absence of the derivative. The distributions of the channel's substates and main states conductances recorded in three independent experiments have illustrated in Fig. 4B. Statistical analyses of conductances in the absence of the derivative showed values of 1839 ± 267 pS, 3116 ± 281 pS and 4240 ± 304 pS for monomer, dimer and trimer, respectively. Corresponding values in the presence of (**5a**) were 1796 ± 286 pS, 3038 ± 315 pS and 4279 ± 304 pS. In other words, there was no significant difference between conductance sizes of monomer, dimer and trimer in the absence and presence of compound (**5a**).

Discussion

Of the derivatives used in this study, compound (**5b**) differs from compound (**5a**) only in one extra CH_2 group. Meanwhile, it showed much stronger inhibition characteristics (MIC = 0.39 μg/ml) compared with (**5a**) (MIC > 6.25 μg/ml) [30] when it was introduced to the *Mycobacterium tuberculosis* culture. Although there might be many potential targets for these derivatives in cell, this study investigated their effects on the OmpF channel activities at a molecular level.

The incorporation of the channel into the model membrane has been shown to be unidirectional [14]. The experiments carried out in this study mimicked the channel activity in the *E. coli* outer membrane. The introduction of the thiadiazole derivatives to the *cis* side would mimic that of happening in native membrane and shows the effects of the thiadiazole derivatives on the channel from the intracellular side. In other words, the effects seen here are considered to be due to thiadiazole derivatives interactions with turns, channel's lumen and constriction zone.

The effects of final products (**5a**, **5b**) on artificial bilayers were studied by voltage clamp technique and did not show any significant effects on the membrane integrity, breaking voltage, capacitance and stability (data not shown). Several *in vitro* studies have

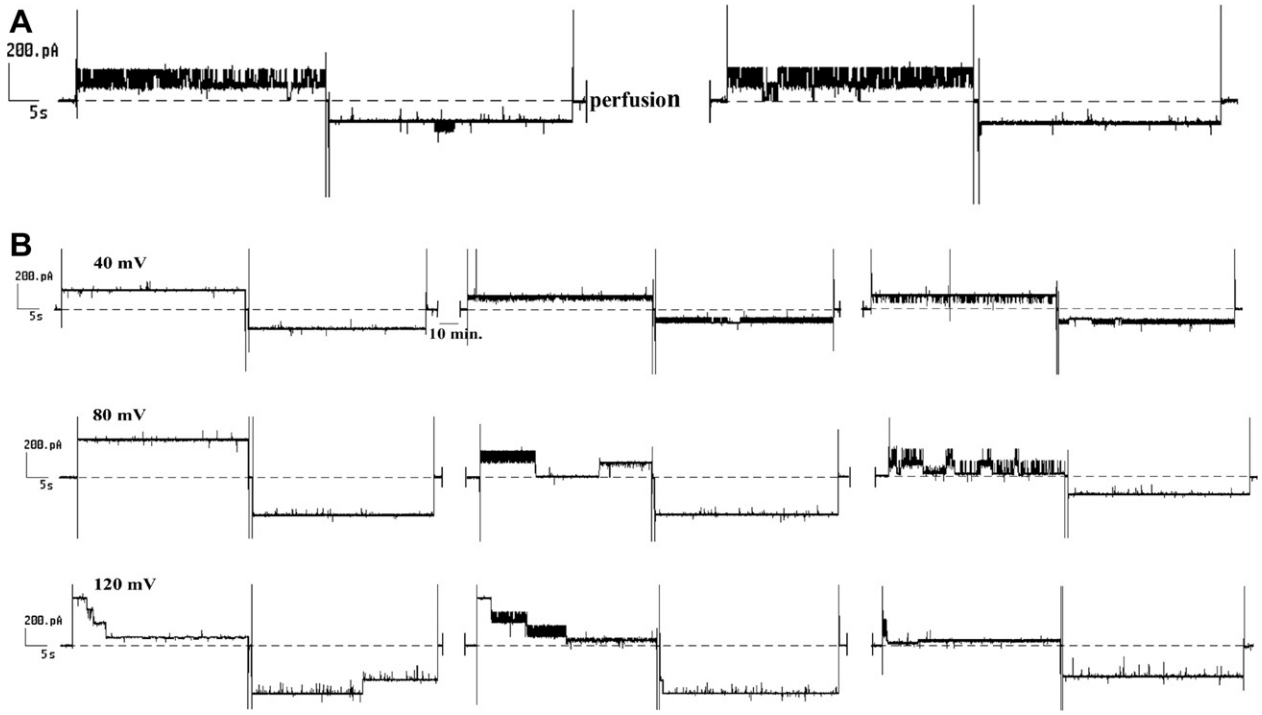

Fig. 3. (A) The effects of removal of propyl ester derivative from *cis* compartments at different polarities. Perfusion cannot eliminate induced effect of the chemical on OmpF behavior. (B) The effects of potential difference on OmpF activity in the presence of propyl ester derivative. Complete closure take place only at high pd.

shown that the derivatives might exert their effects on channel gating through modification of lipid environment [33], causing cell death by disrupting the protein lipid interaction. This supports the hypothesis that addresses the effects of negatively charged lipopolysaccharides and other constituents [34–36] in the formation of the path required for derivatives to cause cell death.

When compound (**5b**) was introduced into the vicinity of the channel lumen (Fig. 2A), both the gating pattern of the channel and its conductance levels changed. Appearance of fast gating, in particular only at negative polarity represents the frequent and transient obstruction of the channel by the derivative [15,16,27]. The alteration might be due to either binding of derivative to the crucial part of the channel involved in gating [17], or simply limiting the electrolyte conducting path of the channel. In the former case, the derivative causes channel malfunction, which might be the mechanism by which it causes bacterial cell death.

The other possibility is the formation of a path to facilitate the rapid uptake of the derivative. Through this uptake path it can enter the cell where it imposes its final antibacterial effect(s). In this study we did not monitored any changes in the membrane permeability following the introduction of derivatives (data not shown). Consequently, although the derivative might enter into membrane and exert its effect on the channel through the membrane, it seams unlikely to form water filed pores by itself.

Appearance of substates with various conductance sizes may correspond to the number of derivatives obstructing the conducting path [37]. We have noticed that these substates are only formed at certain pd's; this shows the coordination of membrane potential and channel structure in fulfilling the solute translocation. This aspect has been discussed in literatures where the membrane potential was found to be effective in the translocation of the colicin E3 through the OmpF channel [38].

The altered gating pattern of the channel persists, even when the free derivatives are removed from the channel medium by perfusion (Fig. 3A). According to the data, some derivative molecules presumably bound to the channel lumen sustain the fast flickering behavior. This was not the case in the control experiments which were carried out in the absence of the derivative. After 20 min, pd's polarity was reversed to positive. Under these conditions unbound chemicals would drawn away from the channel eyelet area and as a consequence the channel opened. However, this was not always the case. On some occasions it was observed that the channel did not fully open and a rather stable partial closure had been established. This suggests the possible binding of chemicals to the channel, obstructing it and/or affecting the voltage sensitive part of it, although we cannot elaborate the actual binding point(s). On the other hand, the current traces indicate that the channel fast gating occurs at both polarities at pd's as low as 40 mV (Fig. 3B). This indicates the failure in complete translocation of the chemicals through the channel, both through the constriction zone or by means of electrophoresis. However, at higher pd's they mainly remained in the vicinity of the channel constriction zone on the *cis* side, and fast flickering behavior of the channel ceased when the pd was increased. This well shows the effect of applied pd in providing enough energy to enable the chemical translocation through the channel.

In conclusion, the different OmpF channel gating behavior observed with propyl ester derivative (**5b**) and ethyl ester derivative (**5a**) could be due to direct binding of these compounds to the channel, obstructing the conducting path and/or indirect effect through interfering with channel's surrounding membrane microenvironment (e.g. membrane fluidity, lateral pressure, charge distribution of the nearby amino acids). Compound (**5b**) shows more significant changes in the channel characteristics than compound (**5a**) that well consistent with *in vitro* experiments. However, more experiments are needed to learn the actual molecular mechanism(s) by which these molecules affect the membrane and channel characteristics.

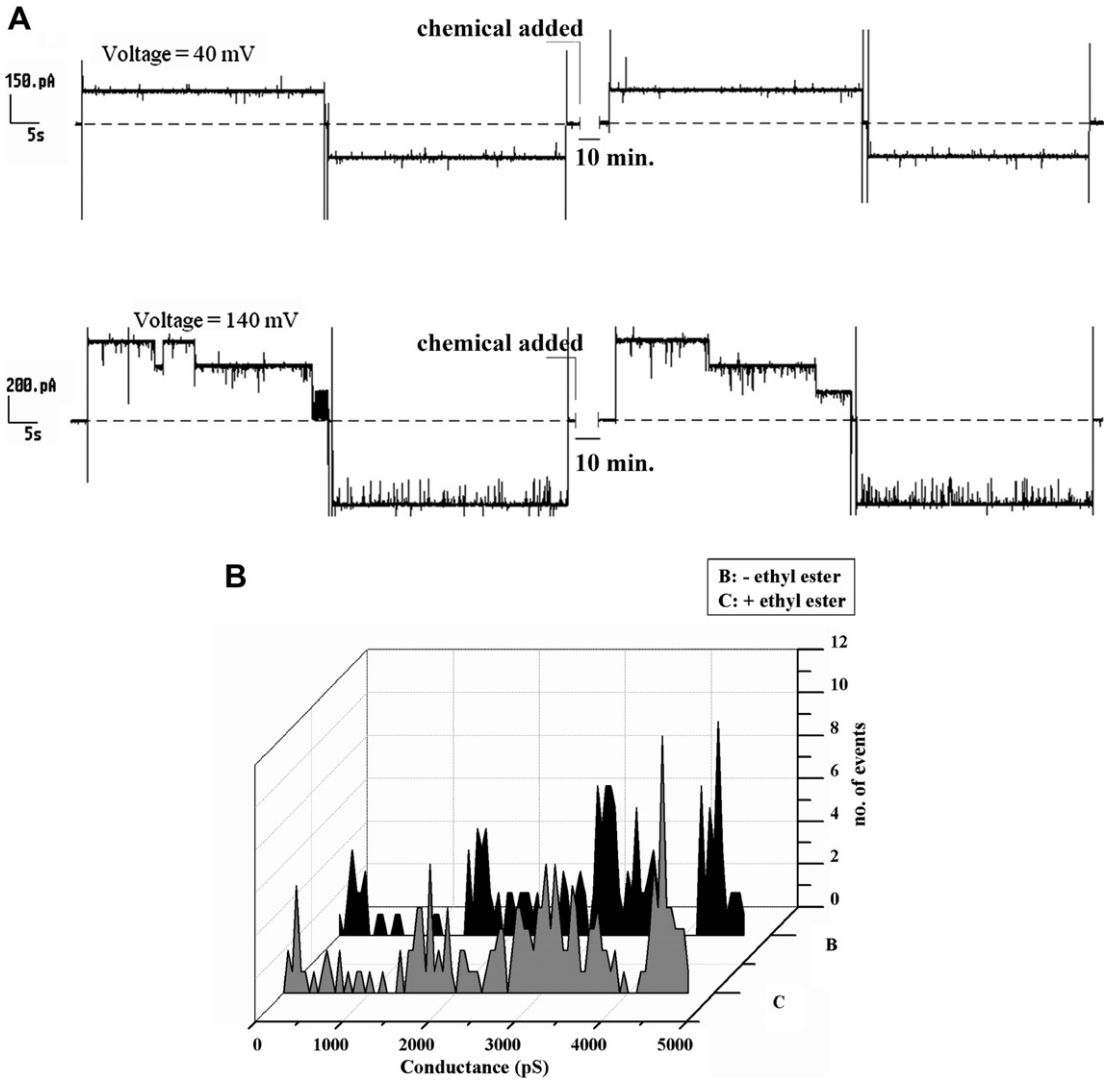

Fig. 4. (A) The effect of ethyl ester derivative on OmpF channel activity. The compound did not change the channel activity pattern or the conductance size of the channel. (B) Conductance distributions of the activities of about 331 channels, recorded in three separate experiments. There is no significant difference between ethyl ester treatment and normal OmpF.

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