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Effect of Entomocidal Proteins from *Bacillus thuringiensis* on Ion Permeability of Apical Membranes of *Tenebrio molitor* Larvae Gut Epithelium

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Abstract—Effects of entomocidal Cry-type proteins, δ-endotoxins Cry3A and Cry11A produced by *Bacillus thuringiensis*, on ion permeability of the apical membranes of intestinal epithelium from *Tenebrio molitor* larvae midgut were studied. Using potential-sensitive dyes safranine O and oxonol VI and ΔpH indicator acridine orange, it was shown that placing brush border membrane vesicles (BBMV) (loaded with Mg^{2+} during their preparation) into a salt-free buffer medium resulted in spontaneous generation of transmembrane electric potential on the vesicular membrane (negative inside the vesicles) accompanied by acidification of the aqueous phase inside the vesicles. The generation of transmembrane ion gradients on the vesicular membrane was a result of an electrogenic efflux of Mg^{2+} from the vesicles as shown by abolishing of the membrane potential by such agents as $MgSO_4$ or $CaCl_2$ in centimolar concentrations, a highly lipophilic cation tetraphenylphosphonium, and some blockers of cell membrane Ca^{2+}-channels in submillimolar concentrations. A passive generation of membrane potential on the vesicular membrane (but positive inside the vesicles) was also observed upon addition of centimolar concentrations of K_2SO_4. Addition of δ-endotoxins Cry3A and Cry11A to the vesicle suspension in a salt-free buffer medium or in the same medium supplemented with centimolar concentrations of K_2SO_4 exerted a pronounced hyperpolarization of the vesicular membrane. This hyperpolarization was sensitive to the same agents, which abolished the membrane potential generation in the absence of δ-endotoxin. It is concluded that Cry proteins induced in BBMV from *T. molitor* opening pores or ion channels, which were considerably more permeable for alkaline- and alkaline-earth metal cations than for the accompanying anions.

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Key words: *Bacillus thuringiensis*, δ-endotoxin Cry3A, *Tenebrio molitor*, BBMV, membrane potential, ion channels, potential-sensitive dyes, membranotropic effect of δ-endotoxin Cry3A

Bacillus thuringiensis is a microorganism producing entomocidal proteins, δ-endotoxins, which kill larvae of many insect species, mainly *Lepidoptera*, *Diptera*, and *Coleoptera*, and also nematodes. The entomocidal proteins are synthesized in a sporulating bacterial cell and produce in it large insertions (crystals) [1]. δ-Endotoxins include two classes of proteins, Cry- and Cyt-types [2]. About a hundred various Cry proteins are now known. They have similar secondary and tertiary structures but are different in the spectra of sensitive insects. Thus, the Cry1 family proteins are toxic for caterpillars of various *Lepidoptera*, whereas δ-endotoxin Cry3A is toxic for larvae of some beetles [1, 2].

As a rule, Cry proteins are prototoxins with molecular weight usually about 130-140 kDa. They are activated by degradation of the C-terminal fragment of the molecule resulting in active toxins with molecular weight of 65-70 kDa [3]. However, some proteins, e.g. Cry3A and Cry11A, initially have molecular weight of 65-70 kDa, and their spatial structure corresponds to that of active toxins from high molecular weight Cry proteins [1]. On entrance and proteolytic activation in the intestine of an insect, the toxins are suggested to bind with a specific receptor on the apical membrane of epithelial cells and

Abbreviations: BBMV, brush border membrane vesicles; TPP^+, tetraphenylphosphonium.

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produce in it pores or ion channels. The toxin is oligomerized immediately before or concurrently with penetration into the membrane [4].

The ability of Cry proteins to form ion channels has been confirmed in experiments on artificial phospholipid membranes [5], brush border membrane vesicles (BBMV) prepared from columnar epithelium from insect larvae [6], and on intact intestinal epithelium preparations [7]. Potential-sensitive dyes, which permit researchers to follow formation of ion channels in the membrane, are used more often [6].

The mechanisms of action of Cry proteins have so far usually been studied on intestinal membrane preparations from caterpillars whose digestion features are fundamentally different of those of larvae of many other insect families. Thus, they are characterized by high pH values of the intestinal fluid, some electrophysiological features of epithelial cells, and also the presence in the intestinal epithelium of specialized goblet cells that play an important role in the energy supply of nutrient transport from the intestinal lumen into hemolymph [8-10]. Nevertheless, studies on the mechanism of Cry toxin action on other economically important insects, e.g. larvae of beetles, were only recently started [11, 12]. The present work is concerned with the effect of δ-endotoxin Cry3A on ion permeability of the apical membrane of intestinal epithelial cells from the yellow mealworm *Tenebrio molitor* larvae, which are sensitive to this protein [13]. The experiments described in this work were performed using potential-sensitive dyes safranine O and oxonol VI and the ΔpH-indicator acridine orange, which is capable of penetrating across biological membranes.

MATERIALS AND METHODS

Materials. Reagents used were as follows: tripcasin (Human, Hungary); yeast extract (Serva, Germany); mannitol, EGTA, BSA, safranine O, acridine orange, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), MES (2-[*N*-morpholino]ethanesulfonic acid), BTP (bis-tris-propane; 1,3-*bis*-*tris*-(hydroxymethyl)methylaminopropane), valinomycin, and $CaCl_2$ (Sigma-Aldrich, USA); oxonol VI (Molecular Probes, USA); verapamil (Orion, Finland); FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) and TPPCl (tetraphenylphosphonium chloride) (Fluka, Switzerland). Other reagents were chemically pure products of domestic production.

Isolation of δ-endotoxins. The following strains of *B. thuringiensis* strains were used: BKPM B-2395 (ssp. *israelensis*) from the Collection of the Institute of Genetics and Selection of Industrial Microorganisms (Moscow) and a ssp. *tenebrionis* strain (Sandoz Crop Protection Corp., USA). The microorganisms were grown at 28°C on liquid medium containing 1% tripcasin, 0.2% yeast extract, and 0.6% glucose until the sporangia were fully lysed [14]. Endotoxin crystals were separated from other components of the cell hydrolysate in a biphasic system xylol–water [14]. δ-Endotoxin Cry11A was isolated from entomocidal crystals of the BKPM B-2395 strain by selective alkaline extraction [15].

To prepare δ-endotoxin Cry3A, crystals of ssp. *tenebrionis* were dissolved in 0.1 M sodium carbonate buffer (pH 10.7) for 1 h at 28°C.

Just before use, both toxins were separated from the peptides and passed into 50 mM sodium carbonate buffer (pH 9.5) on a PD-10 column (GE Healthcare, Sweden).

Preparation of apical membrane vesicles of intestinal epithelium (BBMV) from *T. molitor* larvae. Midguts were isolated from larvae at 4-5 stage using a binocular microscope. The insects were placed onto a glass in a drop of buffer medium that contained 17 mM Tris-HCl (pH 7.5), 0.3 M mannitol, and 5 mM EGTA (buffer A), opened, and the part of the intestine corresponding to the midgut was cut out. The isolated material was washed with buffer A supplemented with 1% (v/v) proteinase inhibitor cocktail for mammalian tissues (Sigma-Aldrich Corp.), placed into a chosen volume of buffer A, and stored at –80°C until use.

To prepare BBMV, the frozen material was thawed and homogenized in buffer A supplemented with proteinase inhibitor cocktail. The membranes were isolated by an approach that included treatment with $MgCl_2$ and differential centrifugation as described in [16]. Judging by specific activities of leucine aminopeptidase, which is a marker enzyme of the apical membrane, the isolated membrane preparation was purified 20-fold as compared to the initial homogenate. Orientation of vesicular membranes in the resulting preparation of BBMV was assessed by sensitivity to Triton X-100 of the leucine aminopeptidase activity located *in vivo* on the external surface of the corresponding cellular membranes. In the presence of 1% Triton X-100, this activity not only did not increase but even slightly decreased, and this indicated that the membrane preparation had virtually no inverted vesicles with latent activity of this marker enzyme. According to some literature data, the average diameter of BBMV calculated from their equilibrium volume in media with different osmolarity should be 350-380 nm.

Determination of protein concentration. The protein concentration was determined by the Bradford method [17] using the Bio-Rad protein assay kit (Bio-Rad Laboratories GmbH, Germany) with BSA as a standard.

Recording of changes in membrane potential and transmembrane pH gradient on the vesicular membrane. Membrane potential generation on the vesicular membrane, negative inside the vesicles, was followed by changes in the absorption difference of the potential-sensitive dye safranine O at 554 and 524 nm ($\Delta A_{554-524}$) [18]. Generation on the same membrane of the opposite polarity membrane potential (positive inside the vesicles) was

recorded by changes in the absorption difference at 590 and 610 nm ($\Delta A_{590-610}$) of another potential-sensitive dye, oxonol VI [19]. The decrease in pH inside the vesicles was followed with the ΔpH indicator acridine orange, which is capable of penetrating across biological membranes [20]. Spectrophotometry was performed at room temperature using a Hitachi-557 spectrophotometer (Japan) in standard 1-cm cuvettes without mixing the specimens during the determinations. The incubation medium of vesicular membranes (1.5 ml) used in these experiments contained 0.25 M mannitol and 10 mM BTP-MES (pH 8.6) or 10 mM HEPES-BTP (pH 7.12). Depending on the measurement performed, the medium was supplemented with 10 μM safranine O, 2 μM oxonol VI, or 12 μM acridine orange. The vesicles were placed into the incubation medium to the final protein concentration of 10 μg/ml protein. Other experimental conditions are shown in the legends to the figures. The figures present representative kinetic curves from at least three independent experiments.

RESULTS

Characteristics of inherent ion permeability of BBMV from *T. molitor* larvae. In the first series of experiments we tested whether the prepared vesicles were capable of maintaining ionic transmembrane gradients on the membrane, i.e. assessed its barrier features with respect to some physiologically important ions. We took into account that during the preparation the vesicles could be loaded with Mg^{2+} and that such type membranes could have a pronounced permeability for this cation [6]. In this case, we could expect the vesicular membrane to be polarized (negative inside the vesicles) upon placing the vesicles into Mg^{2+}-free medium as a result of electrogenic efflux of these ions from the vesicles according to the transmembrane gradient of their electrochemical potential. Data presented in Fig. 1a obtained with safranine O indicated that introduction of BBMV vesicles in a buffer salt-free medium immediately initiated a relatively rapid change in the absorption of this indicator, and this was consistent with the expected polarization of the vesicular membrane. Such interpretation of the observed effect was confirmed, first of all, by its rapid suppression (Fig. 1a) upon the subsequent addition of $MgSO_4$ (20 mM) to the vesicles, that most likely was a result of a decrease (or even disappearance) of the transmembrane Mg^{2+} gradient on the vesicular membrane. The membrane potential on the vesicular membrane was also abolished in response to addition of centimolar concentrations of $CaCl_2$ (Fig. 1a). A rapid dissipation of the signal of safranine O was also observed in the presence of a strongly lipophilic cation, tetraphenylphosphonium (TPP^+), which can depolarize biological membranes (Fig. 1a). The supposed Mg^{2+}-dependent polarization of vesicles was also evidenced by a decrease in pH of their internal aqueous phase upon their placing into a salt-free medium (Fig. 1b), which was easily recorded with acridine orange. The recorded shift in the intravesicular pH seemed to be caused by proton transfer into vesicles, which was energetically supplied by Mg^{2+}-dependent polarization of the vesicular membrane and conditioned by its own proton permeability. This was indicated by a markedly accelerated acidification of the intravesicular medium in the presence of FCCP and also by its rapid inversion upon addition to the vesicles of magnesium sulfate in centimolar concentrations (Fig. 1b) or calcium chloride (data not presented). A significantly

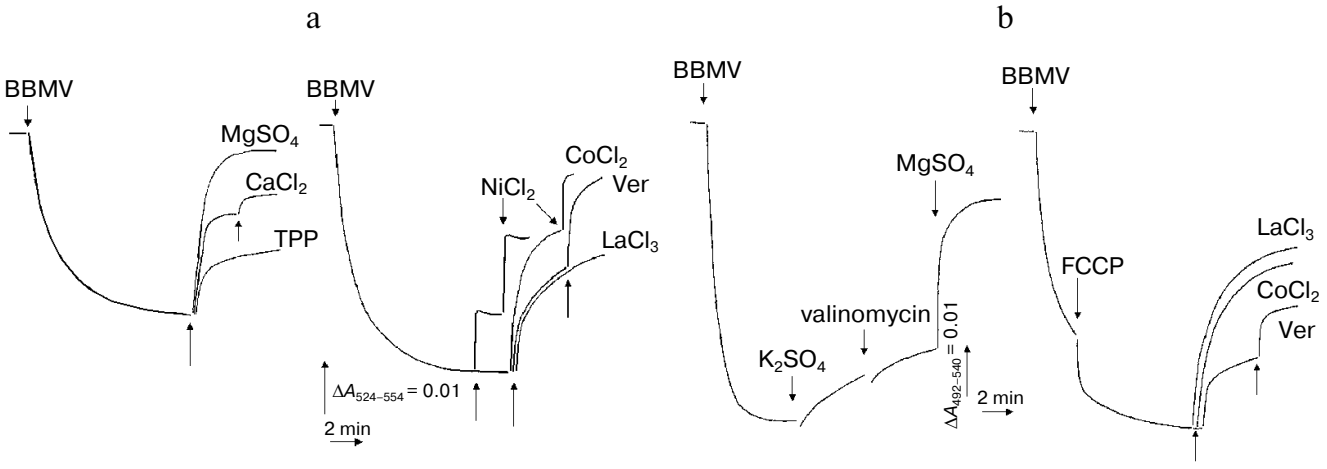

Fig. 1. Spontaneous generation in BBMV of membrane potential (negative inside the vesicles) (a) and of acidification (ΔpH) inside the vesicles (b) during their incubation in a salt-free buffer medium and sensitivity of these processes to centimolar concentrations of $MgSO_4$, K_2SO_4, $CaCl_2$, TPP^+, and blockers of Ca^{2+}-channels of cell membranes. Where indicated: 20 mM $MgSO_4$, K_2SO_4, and $CaCl_2$, 0.6 mM TPPCl, 100 μM $LaCl_3$, $CoCl_2$, $NiCl_2$, and verapamil (Ver), 2 μM FCCP, and 4 μM valinomycin were added to BBMV suspended in buffer (pH 8.6) supplemented with safranine O (a) or acridine orange (b).

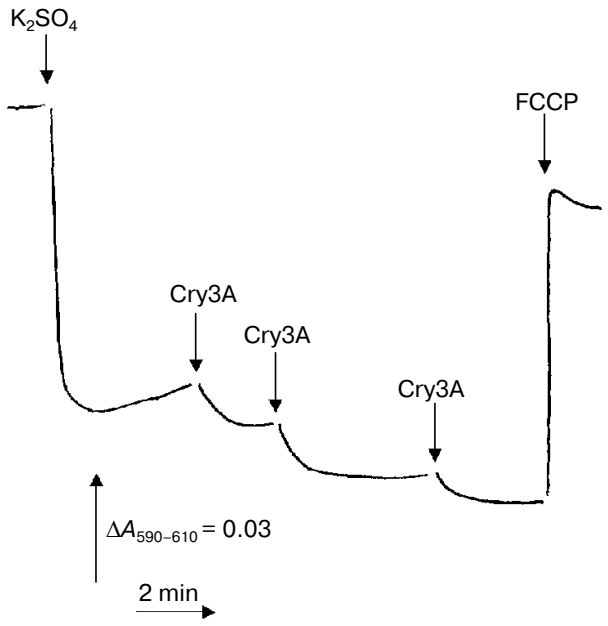

Fig. 2. Generation of K^+ diffusion potential in BBMV (positive inside the vesicles) triggered by addition of centimolar concentrations of K_2SO_4 and the influence of δ-endotoxin Cry3A on this process. Where indicated, 20 mM K_2SO_4, 40 nM Cry3A, and 2 μM FCCP were added to BBMV suspended in salt-free buffer medium (pH 8.6) supplemented with oxonol VI.

slower dissipation of the intravesicular pH shift virtually insensitive to valinomycin was also observed in the presence of equimolar concentrations of potassium sulfate (Fig. 1b), and this seemed to indicate a rather high permeability of the vesicular membrane also for K^+.

These findings indicated that the prepared BBMV could maintain on their membrane both ΔpH and transmembrane electric potential. The latter ability was additionally confirmed by data on the generation on this vesicular membrane of membrane potential with the other polarity, namely the K^+-diffusion potential that is positive inside the vesicles. The generation of this potential under the same experimental conditions was induced by addition to the vesicles of potassium sulfate in centimolar concentrations and could be recorded with oxonol VI (Fig. 2). Note that equimolar concentrations of KCl as differentiated from K_2SO_4 did not induce generation of the K^+-diffusion potential in the vesicles under study (data not presented). This seemed to indicate that the vesicular membrane has a high permeability for Cl^-, which could be comparable with its permeability for K^+.

Then we tried to elucidate whether the ion permeability of BBMV responsible for the observed effects was sensitive to some blockers of ion channels in cell membranes. The membrane potential generation triggered by placing the vesicles into a salt-free buffer medium was very efficiently suppressed in the presence of sub-millimolar concentrations of La^{3+}, Co^{2+}, and Ni^{2+}, and also verapamil, which is an organic blocker of L-type Ca^{2+}-channels (Fig. 1a). All these agents also efficiently reversed the intravesicular medium acidification mediated through the membrane potential generation on the vesicular membrane (negative inside the vesicles) under the same experimental conditions (Fig. 1b).

Modulation of ionic permeability of vesicular membranes under the influence of endotoxins. Addition of endotoxin Cry3A to BBMV suspended in salt-free buffer caused an additional increase in the transmembrane electric potential on the vesicular membrane (negative inside the vesicles), which could be associated with the appearance in it of new ion channels providing for the efflux of intravesicular magnesium ions (Fig. 3a). This effect was augmented with an increase in the toxin concentration. But addition to the vesicles of 20 mM $MgSO_4$ resulted in virtually complete dissipation of the toxin-generated membrane potential. A similar dissipative effect was also caused by addition to the vesicles of centimolar concentrations of $CaCl_2$ (data not presented). The acidification of the intravesicular medium was also increased in the presence of the toxin, and this effect was also reversed upon addition of centimolar concentrations of $MgSO_4$ (Fig. 3b) or $CaCl_2$ (data not presented).

An increase in the membrane potential (positive inside the vesicles) upon addition of 20 mM K_2SO_4 was also found in the presence of Cry3A (Fig. 2). However, no effect of the toxin was recorded in the presence of the

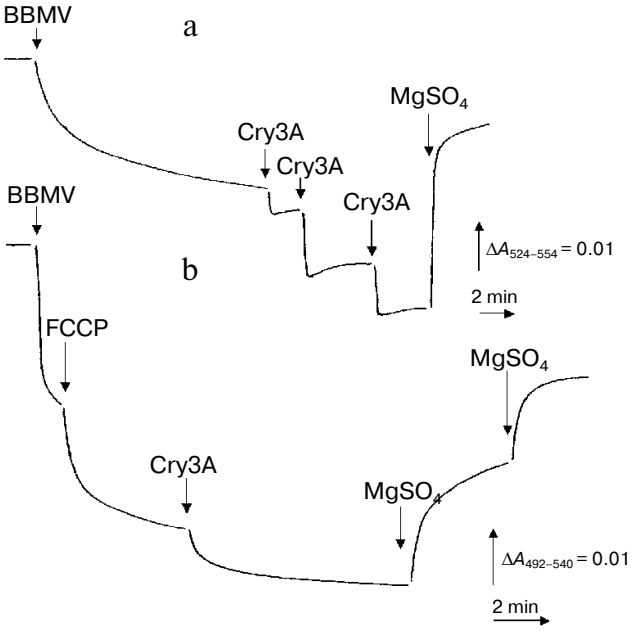

Fig. 3. Hyperpolarization of BBMV (a) and stimulation of acidification inside the vesicles (b) in salt-free buffer medium (pH 8.6) initiated by addition of δ-endotoxin Cry3A to the suspension of vesicles. Where indicated, 40 nM Cry3A, 20 mM $MgSO_4$, and 2 μM FCCP were added to BBMV suspended in buffer (pH 8.6) supplemented with safranine O (a) or acridine orange (b).

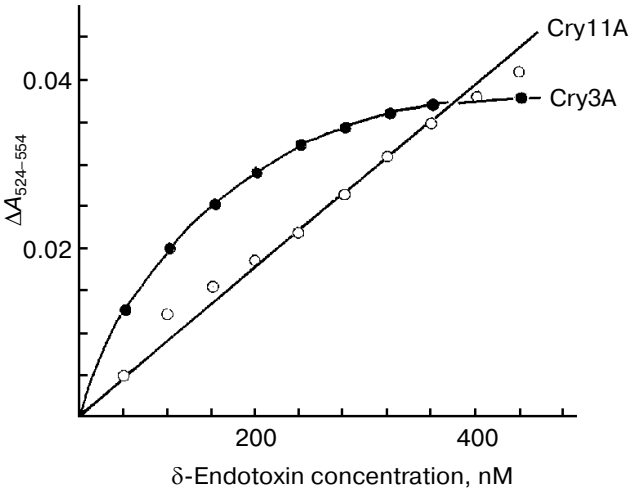

Fig. 4. Dependence of BBMV membrane depolarization induced by Cry3A or Cry11A in salt-free buffer medium (pH 8.6) on δ-endotoxin concentration. For experimental conditions, see Fig. 3 legend.

same concentration of KCl, and this could be associated with a high permeability of the vesicular membrane for K^+ and Cl^-.

Blockers of Ca^{2+}-channels inhibiting the spontaneous generation in BBMV of membrane potential and transmembrane pH gradient displayed in similar concentrations also inhibited the toxin-induced modulation of transmembrane ion gradients (data not presented). But it is still unclear whether the endogenous and toxin-induced in BBMV pores or channels are different in the sensitivity to such type inhibitors.

The effect of endotoxin Cry11A on the ion permeability of BBMV from *T. molitor* was similar to that of Cry3A (data not presented). But, according to data shown in Fig. 4, the dependence of the toxin-induced increase in the membrane potential on the Cry3A concentration was characterized by saturation, whereas in the case of Cry11A the dependence was linear.

Note that a decrease in pH of the incubation medium from 8.6 to 7.12 had virtually no influence on the results of the experiments. The only difference in the behavior of vesicles at neutral pH values was a markedly lower value of the transmembrane gradient of pH spontaneously generated on the vesicular membrane in the absence of salts (data not presented).

DISCUSSION

Ion permeability of BBMV from *T. molitor* larvae. Transmembrane ion transfer plays an important role in functioning of the intestinal epithelium in insects. This problem is best studied on caterpillars. The combined action of the V type ATPase of goblet cells located in the apical membrane [9] and $K^+/2H^+$ antiporter [8, 21] resulted in production of K^+ concentration gradient on both sides of the apical membrane, and this gradient contributed to the symport of amino acids and potassium ions from the intestinal lumen into the cytoplasm across the apical membrane of columnar cells. Passive transport of K^+ occurred only through Ba^{2+}-blocked ion channels in basolateral membranes of epithelial cells, whereas this cation was not transferred across apical membranes [22].

According to these concepts, vesicles prepared from isolated apical membranes of columnar epithelium should have channels for passive ion transport. However, we have observed that such cations as K^+, Mg^{2+}, and Ca^{2+} are capable of penetrating across the vesicular membrane in both directions depending on the direction of their electrochemical potential gradient (Figs. 1-3). Due to the high purification of apical membranes in our preparations (nearly 20-fold), an admixture of basolateral membranes in them is unlikely. Therefore, the cations were likely to be passively transferred due to properties of apical membranes of the intestinal epithelium from *T. molitor* larvae. Studies on ion permeability of membrane of the brush border from larvae using both the patch clamp approach [23] and a potential-sensitive dye, Dis-C_3-(5) [24, 25], also have revealed the presence of their own ion channels. These channels either are poorly cation-selective, or are represented by two types of channels with, respectively, strong cationic and anionic selectivity [23]. The presence of these channels could not be associated with an admixture of basolateral membranes in the preparation under study because Ba^{2+} failed to influence their functioning [23]. Moreover, similar channels were also found in the apical membrane of the intact epithelial layer of intestine from the *Manduca sexta* caterpillar [7]. Normally, the activity of these channels is low but is increased under specific physiological conditions: in the cold, during fasting, and also during the molt [7]. Most likely, ion channels in intestinal epithelium cells are controlled by the organism's regulatory systems, which cannot act in BBMV preparations [23].

Ion transfer in BBMV preparations from insects has usually been studied using K^+ salts [6]; nevertheless, in the present work other cations, Mg^{2+} and Ca^{2+}, were also shown to penetrate across the apical membranes of *T. molitor*. We found that vesicular membranes prepared from *T. molitor* larvae had rather high permeability for K^+ and Cl^-, but displayed pronounced barrier properties for SO_4^{2-}. And, judging by absence of the membrane potential generation on the membranes in the presence of KCl, the difference in their permeability for K^+ and Cl^- seemed to be insufficient to detect membrane polarization using potential-sensitive dyes. But according to the literature, such behavior is not inherent in apical membranes of the intestinal epithelium of caterpillars. Their epithelium is known to actively transfer Cl^- from the intestinal lumen into epithelial cells and further into hemolymph.

However, these anions are mainly transferred across the apical membrane of goblet cells because intensity of their transfer across the apical membrane of columnar epithelium is too low [26]. In spite of a significant permeability of BBMV preparations from caterpillars for potassium and chloride ions [23], the difference in these parameters is sufficient (under different situations, the ratio of the membrane permeabilities for these ions, p(K)/p(Cl), varies from 1.5 to 8.0) for the membrane polarization in KCl solutions [23]. Just this seemed to allow the membrane potential generation to be detected with the dye Dis-C_3-(5) [6, 24, 25]. Thus, the difference in ion permeabilities of the membranes from caterpillars and *T. molitor* larvae could be caused by some physiological features of their transmembrane ion exchange. First, there are no goblet cells in the epithelium of beetle larvae. Second, pH value of the caterpillars' intestinal fluid is high (up to 12) [10], whereas in *T. molitor* larvae the corresponding pH values are 5.2-5.6 in the anterior part of the intestine and 7.8-8.2 in its posterior part [27]. In the latter case, the intestinal fluid pH is unlikely to be regulated via the $K^+/2H^+$ antiporter [8], and Cl^-/HCO_3^- exchange seems to be more important [26, 28, 29]. The latter mechanism can occur because of the presence in the apical membranes of Cl^- channels [29], which could increase their activity under *in vitro* conditions due to absence of the organism's control mechanisms.

The ion permeability of vesicular membranes from *T. molitor* is, in particular, characterized by significant inhibition of their ion-transporting activity by cobalt, nickel, and lanthanum chlorides, as well as by verapamil (Fig. 1). Because all these agents are inhibitors of calcium transfer across cellular membranes [30], the ion transfer pathways detected in the apical membrane of epithelial cells from *T. molitor* and responsible for the observed effects are likely to be also included in the transmembrane transfer of calcium ions.

Ion permeability of toxin-induced ion pores or channels in BBMV from *T. molitor*. Formation of pores or ion channels in the apical membrane of the intestinal epithelium of insects in the presence of Cry proteins has long been postulated to be the most important stage of their toxic action [1]. The membranotropic effect of these proteins has been demonstrated on preparations of these membranes from some members of the *Lepidoptera* family [6, 23-25, 31]. In the present work, we have recorded the membranotropic activity of δ-endotoxin Cry3A in BBMV from *T. molitor* (*Coleoptera* family) as a shift in the membrane potential of either polarity depending on the ion composition of the incubation medium.

Methods for recording the channel-producing ability of Cry proteins are most often based on using the potential-sensitive dye Dis-C_3-(5) [6, 11]. However, this dye interacts with Cry proteins [24] and, thus, can somewhat distort experimental results. In the present work, we have used other potential-sensitive dyes, safranine O and oxonol VI, and thus prevented the possible appearance of such artifacts.

We have found that potassium, magnesium, and calcium ions can pass through pores or channels produced by the Cry3A toxin. This feature of ion channels induced by Cry proteins has also been detected in vesicles of apical membranes of *Lepidoptera* intestine; these channels are permeable for ions of alkaline and alkaline-earth metals [6]. Formation of channels permeable for Ca^{2+} also specifies the toxic effect of Cry1C and Cry1Ac on the insect cell cultures Sf9 and Cf1, respectively [32, 33]. We have not found any effect of Cry3A on the vesicular membrane polarization in the presence of KCl in the incubation medium. This could be due to an insignificant difference in the selectivity to K^+ and Cl^- between the toxin-induced ionic channels and inherent ion transfer systems of the vesicular membrane.

The majority of our experiments were performed with the endotoxin Cry3A, which is active against *T. molitor* larvae [13]. But the endotoxin Cry11A, which is active against mosquito larvae [2, 34], influenced similarly the polarization of the vesicular membrane from *T. molitor* (data not presented). However, the dependence of the Cry3A-induced shift in the membrane potential on the vesicular membrane on the toxin concentration was characterized by saturation, whereas in the case of Cry11A this dependence was linear (Fig. 4). More likely, this suggests the presence in the apical membrane from *T. molitor* of a specific receptor for Cry3A, whereas Cry11A binding with this membrane is nonspecific. Receptors of Cry proteins were found in apical membranes from different representatives of *Lepidoptera* and *Diptera* [1, 4]. In the potato beetle larvae, the Cry3A receptor role was played by ADAM metalloproteinase [12]. It was found that receptors specifically bound with toxins active against insects from which these receptors were isolated but failed to bind with inefficient toxins [1]. The presence of Cry3A receptor along with the absence of Cry11A receptor in the *T. molitor* membranes under study is in correspondence with the above mentioned data. Nevertheless, the lack of binding with the receptor in our experiments did not affect the high ability of Cry11A to produce pores (Fig. 4). This seems to suggest different mechanisms of the membranotropic effects of these toxins. But, notwithstanding the ability to produce channels in BBMV from *T. molitor*, Cry11A was not toxic for larvae of this beetle. The lack of a clear correlation between the toxicity and ability to produce pores has been also shown for other Cry proteins [6]. Thus, Cry1Ac and Cry1C toxins are 10 times different in toxicity to *M. sexta*, but are similar in ability to produce channels in apical membranes of the intestinal epithelium from this insect at pH 7.5 [35, 36]. It seems that the ability of these two Cry proteins to bind with the epithelial membrane and produce ion channels *in vivo* can be significantly different from their *in vitro* behavior. It seems also that asso-

ciation between the channel formation in the epithelium membranes and the insect death is more complex in beetle larvae than in caterpillars. And finally, although Cry3A is the only toxin known lethal for *T. molitor* larvae, it is not very toxic for this insect [13].

In the present work acidification of the vesicle incubation medium from pH 8.6 (at which the majority of determinations were performed) to pH 7.12 had virtually no effect on the studied parameters. It is known that pH and ionic strength of the medium, as well as the presence in it of bivalent cations, can noticeably influence kinetics of Cry protein incorporation into BBMV membrane (due to modulation of electrostatic interactions during the process) [35, 36]. However, these factors only weakly influence the properties of pores produced earlier in the membrane and the resulting toxic effect of endotoxins. Note also that Cry3A and Cry11A seem to be not involved in the transmembrane transfer of H^+ during BBMV acidification in salt-free incubation medium, because in the other case concurrent and oppositely directed flows of H^+ and Mg^{2+} through the toxin-produced pores in the membrane and lack of its hyperpolarization would occur. Although some data suggest that ion channels produced by Cry proteins can also be permeable for H^+ [36], such behavior of toxin-induced ion channels is not universal and can strongly depend on both cell membrane type and the Cry protein interacting with it. Moreover, the inherent proton permeability of the BBMV membrane responsible for the observed acidification of the intravesicular medium could also increase in the presence of Cry proteins.

In conclusion, Cry proteins incorporating into the BBMV membrane from *T. molitor* increase its permeability for ions of alkaline and alkaline-earth metals much more strongly than for the corresponding anions (counter ions), and this results in a pronounced shift of the membrane potential easily recorded with potential-sensitive dyes.

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