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Ectonucleotide Diphosphohydrolase Activities in Hemocytes of Larval *Manduca sexta*

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In this work, we describe the ability of living hemocytes from an insect (*Manduca sexta,* Lepidoptera) to hydrolyze extracellular ATP. In these intact cells, there was a low level of ATP hydrolysis in the absence of any divalent metal (8.24 ± 0.94 nmol of P_i/h × 10^6 cells). The ATP hydrolysis was stimulated by $MgCl_2$ and the Mg^{2+}-dependent ecto-ATPase activity was 15.93 ± 1.74 nmol of P_i/h × 10^6 cells. Both activities were linear with cell density and with time for at least 90 min. The addition of $MgCl_2$ to extracellular medium increased the ecto-ATPase activity in a dose-dependent manner. At 5 mM ATP, half-maximal stimulation of ATP hydrolysis was obtained with 0.33 mM $MgCl_2$. This stimulatory activity was not observed when Ca^{2+} replaced Mg^{2+}. The apparent K_m values for ATP^{-4} and Mg-ATP^{2-} were 0.059 and 0.097 mM, respectively. The Mg^{2+}-independent ATPase activity was unaffected by pH in the range between 6.6 and 7.4, in which the cells were viable. However, the Mg^{2+}-dependent ATPase activity was enhanced by an increase of pH. These ecto-ATPase activities were insensitive to inhibitors of other ATPase and phosphatase activities, such as oligomycin, sodium azide, bafilomycin A_1, ouabain, furosemide, vanadate, sodium fluoride, tartrate, and levamizole. To confirm the observed hydrolytic activities as those of an ecto-ATPase, we used an impermeant inhibitor, DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid), as well as suramin, an antagonist of P_2-purinoreceptors and inhibitor of some ecto-ATPases. These two reagents inhibited the Mg^{2+}-independent and the Mg^{2+}-dependent ATPase activities to different extents. Interestingly, lipopolysaccharide, a component of cell walls of gram-negative bacteria that increase hemocyte aggregation and phagocytosis, increased the Mg^{2+}-dependent ecto-ATPase activity in a dose-dependent manner but did not modify the Mg^{2+}-independent ecto-ATPase activity. © 2000 Academic Press

Key Words: **ectonucleotide diphosphohydrolase; lipopolysaccharide; hemocyte; *Manduca sexta.***

Insect hemocytes are involved in cellular defense mechanisms against invasive microorganisms. They respond to pathogens and parasites through phagocytosis, nodule formation (aggregate of blood cells entrapping particles such as bacteria), and encapsulation (multilayered cellular envelopes around foreign objects that are too large to be phagocytosed by individual hemocytes) (1–3). Hemocytes passively moving in the insect hemocoel encounter foreign particles and respond to extracellular signals that facilitate their recognition and attachment. Charge, hydrophobicity, and carbohydrate or lipid moiety surface of invading organisms influence the extent of its encapsulation by hemocytes (1, 2). The stages of phagocytosis in insects are morphologically similar to those reported for mammalian phagocytic cells (1), for which it has been suggested that surface enzymes participate in the attachment of the parasites (4, 5).

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Cell–cell recognition and adherence are central processes to many fundamental areas of biology. Surface membrane interactions between parasites and their host cells are of critical importance for the survival of the parasite, from both the immunological and physiological viewpoints (5–7). Parasite membrane components may play a role in the uptake of these organisms by mammalian macrophages (4, 5, 8, 9). Plasma membranes contain enzymes whose active sites face the external medium rather than the cytoplasm. The activities of these enzymes, referred to as ectoenzymes, can be measured using cells (10, 11).

Cell membrane ecto-ATPases are integral membrane glycoproteins that are millimolar divalent cation dependent, low-specificity enzymes that hydrolyze all nucleoside triphosphates (12–14). The identity and the function of ecto-ATPases have been reviewed and the nomenclature "E-type ATPases" was proposed to describe these enzymes (12). Their physiological role is still unknown. However, several hypotheses have been suggested, such as (i) protection from cytolytic effects of extracellular ATP (15–17), (ii) regulation of ectokinase substrate concentration (12), (iii) termination of purinergic signaling (18, 19), (iv) involvement in signal transduction (20, 21), and (v) involvement in cellular adhesion (13, 22–25).

Here we show the presence of Mg^{2+}-independent and Mg^{2+}-dependent ecto-ATPase activities on the cell surface of hemocytes of *Manduca sexta.* We characterize the properties of these enzymes and demonstrate the effects of lipopolysaccharide on these ecto-ATPase activities.

MATERIALS AND METHODS

Insects. Larvae from a colony maintained in this laboratory were reared according to Bell and Joachim (26) on a high wheat germ diet (27), using a 16-h light/dark cycle (lights on at 7 A.M.) at 25°C. Animals were synchronized at the end of the fourth larval instar by the appearance of head capsule apolysis (28). The initiation of wandering behavior was detected by the exposure of the dorsal aorta (29).

Bleeding M. sexta larvae. Fifth-instar larvae (day 3) were used for these experiments. Larvae were chilled on ice for 5–15 min and then bled by cutting a proleg. Free-flowing drops of hemolymph from a single larva were collected into 10 ml of chilled anticoagulant saline (AC-saline)[6] containing 8 mM EDTA, 9.5 mM citric acid, and 27 mM sodium citrate, pH 6.6. Hemocytes were pelleted at low speed (100*g*) for 10 min at 4°C. Pellets were resuspended in chilled AC-saline, and hemocytes from different insects were pooled at this stage. They were pelleted again and resuspended in a *Manduca* saline buffer (30) containing 1.7 mM Pipes buffer, pH 6.6, 4 mM NaCl, 40 mM KCl, and 146 mM sucrose. The pellets were washed twice with this buffer and resuspended in the same buffer to use. Cellular viability was assessed before and after incubations by the trypan blue method (31). Hemocytes were observed under a phase microscope to determine cellular integrity and hemocytes were also able to attach and spread on glass slides. The cellular integrity and viability were not affected under the conditions employed here. Protein concentration was determined by the method of Lowry *et al.* (32), using bovine serum albumin as standard.

Ecto-ATPase activity measurements. Intact cells were incubated for 1 h at 30°C with gentle shaking (40 oscillations/min) in 0.2 ml of a mixture containing, unless otherwise specified, 1.7 mM Pipes buffer, pH 6.6, 4 mM NaCl, 40 mM KCl, 146 mM sucrose, 5.0 mM ATP, and 2.0×10^7 cells/ml, which corresponded to 1 mg of protein/ml, in the presence or in the absence of 5.0 mM $MgCl_2$. The ATPase activity was determined by measuring the hydrolysis of [γ-^{32}P]ATP (10^4 Bq/nmol of ATP) (33). The experiments were started by the addition of cells and terminated by the addition of 0.5 ml of a cold mixture containing 0.2 g of charcoal in 1.0 M HCl. The tubes were then centrifuged at 1500*g* for 10 min at 4°C. Aliquots (0.2 ml) of the supernatant containing the released $^{32}P_i$ were transferred to scintillation vials. The ATPase activity was calculated by subtracting the nonspecific ATP hydrolysis measured in the absence of cells. The ATP hydrolysis was linear with time under the assay conditions used and was proportional to the number of cells. In the experiments where other nucleotides were used, the hydrolytic activity measured under the same conditions described above was assayed spectrophotometrically by measuring the release of P_i from the nucleotides (34). The values obtained for ATPase activities measured using both methods (spectrophotometric and radioactive) were exactly the same.

Reagents. All reagents were purchased from Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]ATP was obtained from Amersham Life Science Inc. (Arlington Heights, IL). Deionized distilled water from a MilliQ system of resins (Millipore Corp., Bedford, MA) was used in the preparation of all solutions. Concentrations of free and complexed species at equilibrium were calculated by using an iterative computer program that was modified (35) from that described by Fabiato and Fabiato (36).

Statistical analysis. All experiments were performed in triplicate, with similar results obtained in at least three separate cell suspensions. Apparent K_m and V_{max} values were calculated using a computerized nonlinear regression fit of the data to the Michaelis–Menten equation (14). Statistical significance was determined by Student's t test. Significance was considered as $P < 0.05$.

RESULTS

Hemocytes from *M. sexta* presented two ecto-ATPase activities on their external surface, a Mg^{2+}-independent ATPase activity (activity measured in the absence of any cation added; Fig. 1A) and a Mg^{2+}-dependent ATPase activity (Fig. 1B). At pH 6.6 (pH of the hemolymph) in the absence of Mg^{2+} (1 mM EDTA), hemocytes were able to hydrolyze ATP (8.24 ± 0.94 nmol of P_i/h × 10^6 cells). As can be seen in Fig. 1B, the Mg^{2+}-dependent ecto-ATPase activity (calculated as total [measured in the presence of 5 mM $MgCl_2$] minus basal ecto-ATPase activities [measured in the presence of 1 mM EDTA]) present in these cells hydrolyzed 16.93 ± 1.74 nmol of P_i/h × 10^6 cells. The time course of hydrolysis by the ecto-ATPase present on the external surface of hemocytes of *M. sexta* was linear for at least 90 min for both activities (Fig. 1). Similarly, in assays to determine the influence of cell density, Mg^{2+}-independent, as well as Mg^{2+}-dependent, activities measured over 60 min were linear over a nearly sixfold range of cell densities (Fig. 2). To check the possibility that the

[6] Abbreviations used: AC-saline, anticoagulant saline; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; LPS, lipopolysaccharide.

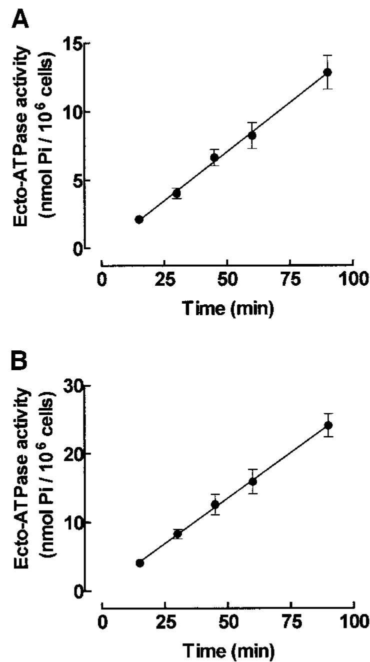

FIG. 1. Time course of hemocyte ecto-ATPase activities. Cells (2.0×10^7 cells/ml) were incubated at 30°C in the reaction medium (final volume 0.2 ml) containing 1.7 mM Pipes buffer, pH 6.6, 4 mM NaCl, 40 mM KCl, 146 mM sucrose, and 5 mM ATP, without (A) or with (B) the addition of 5 mM $MgCl_2$. Data are means ± SE of three determinations with different cell suspensions.

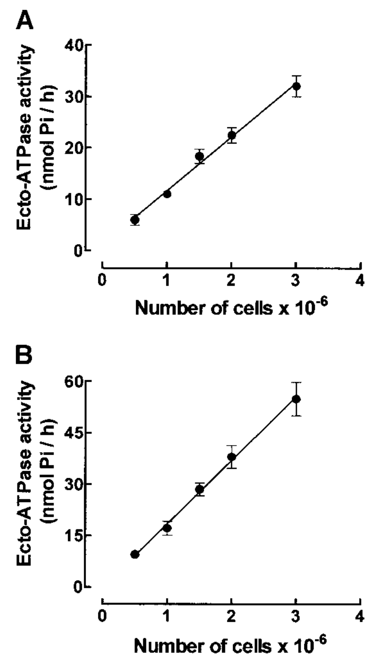

FIG. 2. Cell density dependence of hemocyte ecto-ATPase activities. Cells were incubated for 1 h at 30°C in the same reaction medium (final volume 0.2 ml) described in Fig. 1, without (A) or with (B) the addition of 5 mM $MgCl_2$. Data are means ± SE of three determinations with different cell suspensions.

observed ATP hydrolysis was the result of secreted soluble enzymes, as seen in other cells (37, 38), we prepared a reaction mixture with cells that were incubated in the absence of ATP. Subsequently, the suspension was centrifuged to remove cells and the supernatant was checked for ATPase activity. This supernatant failed to show ATP hydrolysis either in the absence or in the presence of $MgCl_2$ (data not shown).

In the pH range from 6.6 to 7.4, where the cells were alive throughout the time course of reaction, pH had no effect on the Mg^{2+}-independent ATPase activity (Fig. 3). However, the Mg^{2+}-dependent ATPase activity in this pH range progressively increased to reach a maximal level at pH 7.4 (Fig. 3). Other E-type ATPases are also stimulated by an increase of pH (14, 39). To exclude the possibility that the ATP hydrolysis was due to phosphatase or other type of ATPase, different inhibitors for those enzymes were tested. Table I shows that sodium fluoride (NaF) and tartrate, two potent inhibitors of acid phosphatase (14, 40–42), did not inhibit either ecto-ATPase activity. Levamizole, a specific inhibitor of alkaline phosphatases (43, 44), also failed to inhibit the ATP hydrolysis catalyzed by intact hemocytes. The ATPase activities were insensitive to oligomycin and sodium azide, two inhibitors of mitochondrial Mg-ATPase (14); bafilomycin A_1, a V-ATPase inhibitor (45); ouabain, a Na^+/K^+-ATPase inhibitor (46); furosemide, a Na^+-ATPase inhibitor (47); and vanadate, which is a potent inhibitor of P-ATPases (14, 47). The ATP hydrolysis described here might be due to an ATP-diphosphohydrolase activity, as it was inhibited by high concentrations of ADP and AMP (Table I) (48).

Since we used intact cells for measuring the enzyme activities in all the experiments done in this work, it is

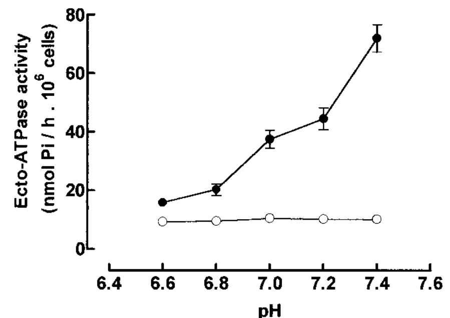

FIG. 3. Effects of pH on hemocyte ecto-ATPase activities. Cells (2.0×10^7 cells/ml) were incubated for 1 h at 30°C in the reaction medium (final volume 0.2 ml) containing 4 mM NaCl, 40 mM KCl, 146 mM sucrose, 5 mM ATP, and 1.7 mM Pipes buffer, adjusted to pH values between 6.6 and 7.4. In this pH range (6.6–7.4), the cells were viable throughout the course of the experiments. The assays were performed with (●) or without (○) the addition of 5 mM $MgCl_2$. Data are means ± SE of three determinations with different cell suspensions.

TABLE I

Influence of Various Agents on Hemocyte Ecto-ATPase Activities

Additions	Relative activity (%)	
	Independent of $MgCl_2$	Dependent on $MgCl_2$
Control	100.0 ± 10.4	100.0 ± 9.1
Levamizole (1.0 mM)	108.8 ± 9.3	95.6 ± 7.9
Vanadate (1.0 mM)	105.8 ± 11.6	99.1 ± 10.1
Tartrate (10.0 mM)	106.2 ± 8.3	93.7 ± 7.9
NaF (10.0 mM)	99.1 ± 9.8	99.9 ± 11.2
Bafilomycin A_1 (10 μM)	93.2 ± 11.7	92.2 ± 8.6
Azide (10.0 mM)	89.5 ± 8.1	108.2 ± 12.3
Oligomycin (10 μg/ml)	103.5 ± 12.1	109.8 ± 10.2
Ouabain (1 mM)	97.2 ± 8.9	112.6 ± 9.8
Furosemide (10 mM)	100.9 ± 7.8	94.7 ± 11.5
AMP (10.0 mM)	63.8 ± 6.1	65.9 ± 7.9
ADP (10.0 mM)	50.6 ± 6.7	54.1 ± 4.3

Note. Ecto-ATPase activities were measured in the standard assay described under Materials and Methods. Activities are expressed as percentages of that measured under control conditions, i.e., without other additions. The Mg^{2+}-independent ATPase (9.4 ± 0.7 nmol of P_i/h × 10^6 cells) and the Mg^{2+}-dependent ATPase (18.9 ± 2.3 nmol of P_i/h × 10^6 cells) activities were taken as 100%. The standard errors were calculated from the absolute activity values of three experiments with different cell suspensions and converted to percentage of the control values.

likely that the described activities are ecto-ATPases. To confirm this, we applied the criterion that an authentic ectoenzyme should be inhibited by an added extracellular impermeant inhibitor (10, 14, 39, 49) such as 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) (14, 39, 49) and possibly by an ecto-ATPase inhibitor, such as suramin, which is also an antagonist of P_2-purinergic receptors (50, 51). Figure 4 shows that DIDS inhibited both ecto-ATPase activities in a dose-dependent manner and that 1 mM DIDS inhibited 43% of the Mg^{2+}-independent ecto-ATPase activity (Fig. 4A) and 87% of the Mg^{2+}-dependent ecto-ATPase activity (Fig. 4B). We found that 2 mM suramin inhibited 53% of the independent ecto-ATPase activity (Fig. 5A), while it totally inhibited the Mg^{2+}-dependent ecto-ATPase activity (Fig. 5B).

Ecto-ATPases are glycoproteins typically stimulated by Mg^{2+} or Ca^{2+} (12–14, 39, 52), although in *Xenopus* oocytes a Mg^{2+}/Ca^{2+}-independent ecto-ATPase activity has been reported (51). For *M. sexta* hemocytes, addition of $MgCl_2$ to the extracellular medium increased the ecto-ATPase activity in a dose-dependent manner (Fig. 6). At 5 mM ATP, half stimulation of ATP hydrolysis was obtained with 0.33 mM $MgCl_2$ (Fig. 6). This stimulatory activity was not observed when Mg^{2+} was replaced by Ca^{2+} (Fig. 6). These data could indicate that the substrate for the Mg^{2+}-independent enzyme is ATP^{4-} whereas that for the Mg^{2+}-dependent enzyme is $Mg\text{-}ATP^{2-}$. The apparent K_m values for ATP^{-4} (Fig. 7A) and $Mg\text{-}ATP^{2-}$ (Fig. 7B) were 0.059 and 0.097 mM, respectively.

We analyzed the specificity of these ecto-ATPase activities for other nucleotides. Table II shows that ATP was the best substrate for these enzymes. The Mg^{2+}-independent ecto-ATPase hydrolyzed UTP, TTP, and ITP at high rates. GTP, CTP, and ADP produced lower reaction rates. ATP was also the best substrate for the Mg^{2+}-dependent activity, although UTP was also hydrolyzed at a high rate. Other nucleotides such as GTP, CTP, TTP, and ITP were poorer substrates (Table II). The Mg^{2+}-dependent enzyme was also able to hydrolyze ADP.

Lipopolysaccharide (LPS), a component of the cell walls of gram-negative bacteria, affects the antibacterial activities of insect hemocytes (1). LPS increases hemocyte aggregation and phagocytosis (3). The physiological role of the ecto-ATPases is still unknown; however, a possible involvement in cellular adhesion has been suggested (13, 22–25). For these reasons, we examined the effect of LPS on the ecto-ATPase activities of hemocytes and found that LPS stimulated the Mg^{2+}-dependent ATPase activity in a dose-dependent manner but had no effect on Mg^{2+}-independent ATPase activity (Fig. 8).

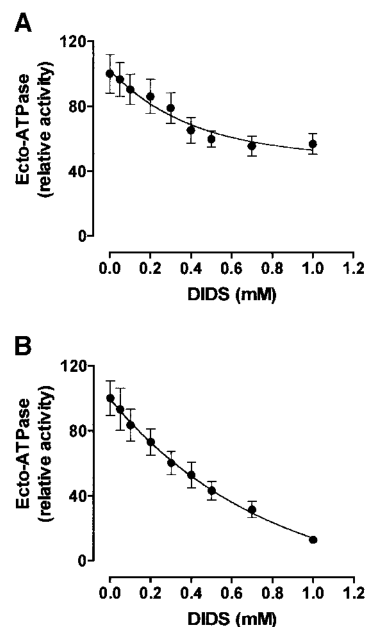

FIG. 4. Effects of increasing concentration of DIDS on hemocyte ecto-ATPase activities. Cells were incubated for 1 h at 30°C in the same reaction medium (final volume 0.2 ml) described in Fig. 1, with the concentrations of DIDS shown on the abscissa. The Mg^{2+}-independent ATPase (8.3 ± 0.9 nmol of P_i/h × 10^6 cells, A) and the Mg^{2+}-dependent ATPase (15.2 ± 1.6 nmol of P_i/h × 10^6 cells, B) activities were taken as 100%. The standard errors were calculated from the absolute activity values of three experiments with different cell suspensions and converted to percentage of the control value.

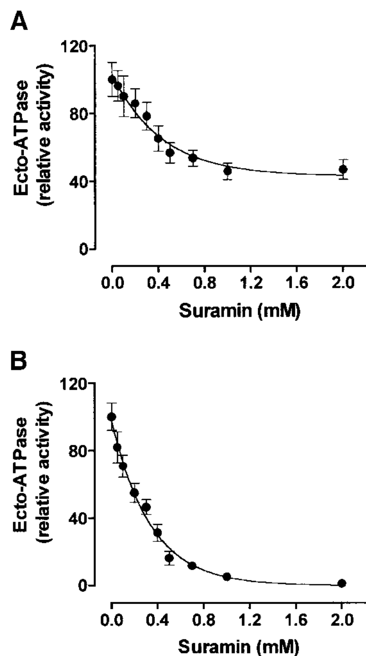

FIG. 5. Effects of increasing concentration of suramin on hemocyte ecto-ATPase activities. Cells were incubated for 1 h at 30°C in the same reaction medium (final volume 0.2 ml) described in Fig. 1, with the concentrations of suramin shown on the abscissa. The Mg^{2+}-independent ATPase (8.3 ± 0.9 nmol of P_i/h × 10^6 cells, A) and the Mg^{2+}-dependent ATPase (15.2 ± 1.6 nmol of P_i/h × 10^6 cells, B) activities were taken as 100%. The standard errors were calculated from the absolute activity values of three experiments with different cell suspensions and converted to percentage of the control value.

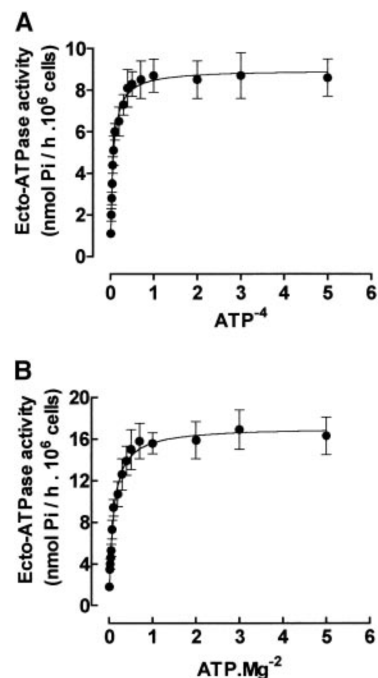

FIG. 7. Dependence of hemocyte ecto-ATPase activities on ATP^{4-} or $Mg\text{-}ATP^{2-}$ concentrations. Cells were incubated at 30°C in the same reaction medium (final volume 0.2 ml) described in Fig. 1, which corresponds to ATP^{4-} (A) or $Mg\text{-}ATP^{2-}$ (B) concentrations varying as shown on the abscissa. Curves represent the fit of experimental data by nonlinear regression using the Michaelis–Menten equation as described under Materials and Methods. The total amounts of ATP and $MgCl_2$ necessary to form the desired ATP^{4-} (A) and $Mg\text{-}ATP^{2-}$ (B) concentrations were calculated as described under Materials and Methods. Data are means ± SE of three determinations with different cell suspensions.

DISCUSSION

In this paper, we report the characterization of Mg^{2+}-independent and Mg^{2+}-dependent ecto-ATPase activities present on the external surface of insect hemocytes. Cellular integrity and viability were assessed, before and after the reactions, by the trypan blue method (22). The integrity of the cells was not affected by any of the conditions used in the assays. The external location of the ATP-hydrolyzing site is supported by its sensitivity to the impermeant inhibitor DIDS (Fig. 4) (14, 39, 49) and to suramin (Fig. 5), which is a noncompetitive inhibitor of ecto-ATPases and an antagonist of P_2-purinoreceptors, which mediate the physiological functions of extracellular ATP (50, 51). Also, a battery of inhibitors for other ATPases that have intracellular ATP binding sites showed no effect on the ATPase activities, either in the absence or in the presence of Mg^{2+} (Table I). For these reasons, we assign an ectolocalization of the ATPase activities described here (10–14).

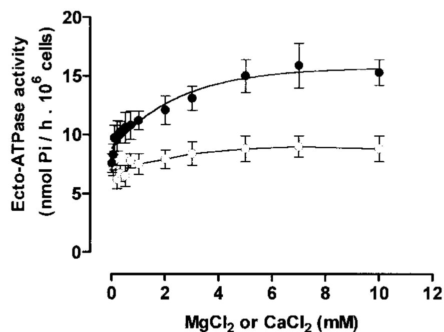

FIG. 6. Dependence of hemocyte ecto-ATPase activities on $MgCl_2$ or $CaCl_2$ concentrations. Cells were incubated for 1 h at 30°C in the same reaction medium (final volume 0.2 ml) described in Fig. 1, with the concentrations of $MgCl_2$ or $CaCl_2$ shown on the abscissa. Data are means ± SE of three determinations with different cell suspensions.

Ecto-ATPases have been described in several vertebrate cell types (10, 12, 37), including blood cells (12, 53, 54) such as macrophages (55, 56), leukocytes (57), and lymphocytes (15, 31, 58–61). Here we characterize the insect hemocyte ecto-ATPase activities, showing that the addition of $MgCl_2$ to the extracellular medium increased the ecto-ATPase activity in a dose-dependent manner (Fig. 6) and that $CaCl_2$ is not able to replace

TABLE II

Substrate Specificity of Hemocyte Ecto-ATPase Activities

	Relative activity (%)	
Nucleotides	Independent of $MgCl_2$	Dependent on $MgCl_2$
ATP	100.0 ± 9.6	100.0 ± 8.1
ADP	56.8 ± 6.6	45.6 ± 3.9
CTP	59.5 ± 4.7	39.3 ± 3.6
GTP	66.2 ± 8.3	43.7 ± 6.9
ITP	79.1 ± 6.8	19.9 ± 1.2
TTP	83.2 ± 9.7	32.2 ± 3.6
UTP	83.5 ± 8.1	82.2 ± 6.3

Note. The ecto-ATPase activities were measured at 30°C in medium containing the nucleotides listed (5 mM), 1.7 mM Pipes buffer, pH 6.6, 4 mM NaCl, 40 mM KCl, and 146 mM sucrose, and 2.0×10^7 cells/ml in the presence or in the absence of 5.0 mM $MgCl_2$. The Mg^{2+}-independent ATPase (8.7 ± 0.8 nmol of P_i/h × 10^6 cells) and the Mg^{2+}-dependent ATPase (16.2 ± 1.3 nmol of P_i/h × 10^6 cells) activities were taken as 100%. The standard errors were calculated from the absolute activity values of three experiments with different cell suspensions and converted to percentage of the control value. In these experiments, P_i release from all nucleotides, including ATP, was measured using a colorimetric assay as described under Materials and Methods.

$MgCl_2$. Similar results were also obtained by Dombrowski *et al.* (60), who showed that immortalized B lymphocyte ecto-ATPases are Mg^{2+}-dependent enzymes. Regarding the kinetic characteristics of the ecto-ATPase activities described here (Figs. 7A and 7B), the ecto-ATPases present in lymphocytes from different B-cell lines (58, 60) showed a similar value range of K_m for ATP (5–150 μM). In addition, the ecto-ATPase activities from the *M. sexta* hemocytes described here as well as the ecto-ATPase from the vertebrate lymphocyte (58) hydrolyze other nucleoside triphosphates as well as ATP (Table II). However, in contrast with ecto-ATPases described in lymphocytes, in *M. sexta* hemocytes a Mg^{2+}-independent enzyme is present. The ADP hydrolysis was 57 and 46% of the ATP hydrolysis for the Mg^{2+}-independent and the Mg^{2+}-dependent activities, respectively (Table II). Furthermore, ADP inhibited the two activities by about 50% (Table I). These data are highly suggestive that the ATP hydrolysis characterized here is due to ecto-nucleotide diphosphohydrolase activity, as already described in other cells (62–66).

There are several types of surface-located enzymes that are able to hydrolyze extracellular nucleotides (65, 68). These enzymes include the ecto-alkaline phosphatases, which hydrolyze nucleoside 5′-tri, 5′-di-, and 5′-monophosphates; ecto-5′-nucleotidase, which catalyzes the hydrolysis of nucleotide 5′-monophosphates; ecto-phosphodiesterase/pyrophosphatase, PC-1, or the phosphodiesterase/nucleotide pyrophosphatase (PDNP) family, which act on phosphodiester bonds of nucleotides, hydrolyzing nucleoside 5′-triphosphates such as ATP to AMP and PP_i, nucleoside 5′-diphosphates such as ADP to AMP and P_i, and diadenosine polyphosphates; and CD39-family ectonucleotide diphosphohydrolase, which was first sequenced and latter identified as ecto-ATP diphosphohydrolase or apyrase (63). CD39 was originally identified on the surface of Epstein–Barr virus transformed B cells and was subsequently shown to be present on activated B and natural killer (NK) cells and subsets of activated T cells, but not on resting lymphoid cells (67, 68). Wang and Guidotti discovered that CD39 has sequence homology with a potato apyrase and that CD39 has apyrase activity (63). This work led to the identification of a family of ecto-ATPases that are related in sequence but vary in their membrane topology and tissue distribution (12, 65, 68). The first member of this group, CD39, is able to hydrolyze either ATP or ADP and was called ecto-ATP diphosphohydrolase (65, 66, 68). Further work has shown that some members of this family have a strong preference for ATP, whereas others also efficiently hydrolyze ADP (65, 66, 68). Differences in the response to pH variation observed for the two ectonucleotide diphosphohydrolases described here (Fig. 3), as well as in their sensitivity to the inhibition by DIDS (Fig. 4) and suramin (Fig. 5) and to the stimulation by LPS (Fig. 8), suggest that these activities are due to different enzymes.

Bacterial lipopolysaccharide is a potent and pleiotropic stimulus of immune cells, both *in vitro* and *in vivo* (69, 70). In vertebrate blood cells, the biological responses to LPS involve the increase of adhesion capacity (70), and in insect hemocytes, LPS increases aggregation and phagocytosis (3). When we investigated the possible effect of LPS on both ecto-ATPases present in insect hemocytes, it stimulated the Mg^{2+}-dependent ecto-ATPase activity in a dose-dependent manner but

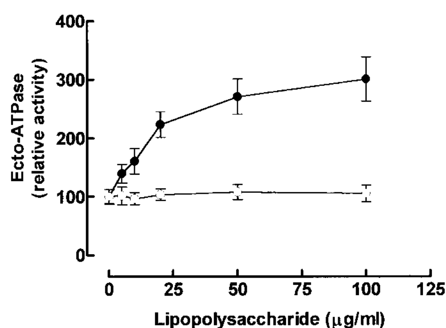

FIG. 8. Effects of increasing concentration of lipopolysaccharide on hemocyte ecto-ATPase activities. Cells were incubated for 1 h at 30°C in the same reaction medium (final volume 0.2 ml) described in Fig. 1, with the concentrations of lipopolysaccharide shown on the abscissa. The Mg^{2+}-independent ATPase (8.7 ± 0.8 nmol of P_i/h × 10^6 cells, open circles) and the Mg^{2+}-dependent ATPase (16.2 ± 1.3 nmol of P_i/h × 10^6 cells, closed circles) activities were taken as 100%. The standard errors were calculated from the absolute activity values of three experiments with different cell suspensions and converted to percentage of the control value.

did not modify the Mg^{2+}-independent ecto-ATPase activity (Fig. 8). In mammalian endothelial cells, LPS treatment modifies pH- and cation-dependent ecto-ATPase activity (71), increasing the Mg^{2+}-dependent ecto-ATPase activity (71). Invertebrates are able to recognize nonself molecules from bacterial cell walls like LPS from the outer membrane of gram-negative bacteria (72). Recognition of LPS is an important function of innate immunity and may have profound consequences for the host. Recently, a group of molecules belonging to the Toll receptor family involved in recognition in *Drosophila melanogaster* has been described (73). In mammals, several homologs of the Toll gene exist, called Toll-like receptor (TLR) proteins, involved in LPS recognition. These receptors mediate the mammalian innate immune response, with different receptors apparently responsible for different classes of pathogens (73). It remains to be elucidated if LPS is directly recognized by the Mg^{2+}-dependent ecto-ATPase on *M. sexta* hemocytes or if the enzyme is activated following the recognition of LPS by other molecules.

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REFERENCES

1. Lackie, A. M. (1988) *Adv. Insect Physiol.* **21,** 85–178.
2. Baines, D., and Downer, R. G. H. (1994) *Arch. Insect Biochem. Physiol.* **26,** 249–261.
3. Lanz-Mendoza, H., Betencourt, R., Fabbri, M., and Faye, I. (1996) *Cell. Immunol.* **169,** 47–54.
4. Vannier-Santos, M. A., Martiny, A., Meyer-Fernandes, J. R., and De Souza, W. (1995) *Eur. J. Cell Biol.* **67,** 112–119.
5. Martiny, A., Vannier-Santos, M. A., Borges, V. M., Meyer-Fernandes, J. R., Asseruy, J., Cunha e Silva, N. L., and De Souza, W. (1996) *Eur. J. Cell Biol.* **71,** 206–215.
6. Alexander, J., and Russel, D. G. (1992) *Adv. Parasitol.* **31,** 175–254.
7. Martiny, A., Meyer-Fernandes, J. R., De Souza, W., and Vannier-Santos, M. A. (1999) *Mol. Biochem. Parasitol.* **102,** 1–12.
8. Handman, E., and Goding, J. W. (1985) *EMBO J.* **4,** 329–336.
9. Russel, D. G., and Wilhem, H. (1986) *J. Immunol.* **136,** 2613–2630.
10. De Pierre, J. W., and Karnovsky, M. L. (1973) *J. Cell Biol.* **56,** 275–303.
11. De Pierre, J. W., and Karnovsky, M. L. (1974) *J. Biol. Chem.* **249,** 7111–7120.
12. Plesner, L. (1995) *Int. Rev. Cytol.* **158,** 141–214.
13. Kirley, T. L. (1997) *J. Biol. Chem.* **272,** 1076–1081.
14. Meyer-Fernandes, J. R., Dutra, P. M. L., Rodrigues, C. O., Saad-Nehme, J., and Lopes, A. H. C. S. (1997) *Arch. Biochem. Biophys.* **341,** 40–46.
15. Filippini, A., Taffs, R. E., Agui, T., and Sitkovsky, M. V. (1990) *J. Biol. Chem.* **265,** 334–340.
16. Zanovello, P., Bronte, V., Rosato, A., Pizzo, P., and Di Virgilio, F. (1990) *J. Immunol.* **145,** 1545–1550.
17. Steinberg, T. H., and Di Virgilio, F. (1991) *Curr. Opin. Immunol.* **3,** 71–75.
18. Weisman, G. A., Turner, J. T., and Fedan, J. S. (1996) *J. Pharmacol. Exp. Ther.* **277,** 1–9.
19. Westfall, T. D., Kennedy, C., and Sneddon, P. (1997) *Eur. J. Pharmacol.* **329,** 169–173.
20. Margolis, R. N., Schell, M. J., Taylor, P., and Hubbard, A. L. (1990) *Biochem. Biophys. Res. Commun.* **166,** 562–566.
21. Yagi, K., Nishino, I., Eguchi, M., Kitagawa, M., Miura, Y., and Mizoguchi, T. (1994) *Biochem. Biophys. Res. Commun.* **203,** 1237–1243.
22. Aurivillus, M., Hansen, O. C., Lazrek, M. B. S., Bock, E., and Obrink, B. (1990) *FEBS Lett.* **264,** 267–269.
23. Cheung, P. H., Luo, W., Qui, Y., Zhang, X., Earley, K., Millirons, P., and Lin, S.-H. (1993) *J. Biol. Chem.* **268,** 24303–24310.
24. Dzhandzhugazyan, K., and Bock, E. (1993) *FEBS Lett.* **336,** 279–283.
25. Stout, J. G., Strodel, R. S., and Kirley, T. L. (1995) *J. Biol. Chem.* **270,** 11845–11850.
26. Bell, R. A., and Joachim, F. G. (1976) *Ann. Entomol. Soc. Am.* **69,** 365–373.
27. Reinecke, J. P., Buckner, J. S., and Grugel, S. R. (1980) *Biol. Bull. (Woods Hole, Mass.)* **158,** 129–140.
28. Truman, J. W., Riddiford, L. M., and Safranek, L. (1973) *J. Insect Physiol.* **19,** 195–203.
29. Prassad, S. V., Ryan, R. O., Law, J. H., and Wells, M. A. (1986) *J. Biol. Chem.* **261,** 558–562.
30. Willott, E., Trenczeck, T., Thrower, L. W., and Kanost, M. R. (1994) *Eur. J. Cell Biol.* **65,** 417–423.
31. Barankiewicz, J., Dosch, H.-M., and Cohen, A. (1988) *J. Biol. Chem.* **263,** 7094–7098.
32. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
33. Sola-Penna, M., Vieyra, A., and Meyer-Fernandes, J. R. (1994) *Z. Naturforsch. C* **49,** 141–146.
34. Lowry, O. H., and Lopez, J. (1946) *J. Biol. Chem.* **162,** 421–428.
35. Sorenson, M. M., Coelho, H. S. L., and Reuben, J. P. (1986) *J. Membr. Biol.* **90,** 219–230.
36. Fabiato, A., and Fabiato, F. (1979) *J. Physiol. (Paris)* **75,** 423–445.
37. Bermudes, D., Peck, K. R., Afifi, M. A., Beckers, C. J. M., and Joiner, K. A. (1994) *J. Biol. Chem.* **269,** 29252–29260.
38. Smith, T. M., Kirley, T. L., and Hennessey, T. M. (1997) *Arch. Biochem. Biophys.* **337,** 351–359.
39. Knowles, A. F. (1988) *Arch. Biochem. Biophys.* **263,** 264–271.
40. Fernandes, E. C., Meyer-Fernandes, J. R., Silva-Neto, M. A. C., and Vercesi, A. E. (1997) *Z. Naturforsch. C* **52,** 351–358.
41. Furuya, T., Zhong, L., Meyer-Fernandes, J. R., Lu, H.-G., Moreno, S. N. J., and Docampo, R. (1998) *Mol. Biochem. Parasitol.* **92,** 339–348.
42. Dutra, P. M. L., Rodrigues, C. O., Jesus, J. B., Lopes, A. H. C. S., Souto-Padrón, T., and Meyer-Fernandes, J. R. (1998) *Biochem. Biophys. Res. Commun.* **253,** 164–169.
43. Van Belle, H. (1972) *Biochim. Biophys. Acta* **289,** 158–168.
44. Van Belle, H. (1976) *Clin. Chem.* **22,** 972–976.
45. Bowman, E. J., Siebers, A., and Altendorf, K. (1988) *Proc. Natl. Acad. Sci.* **85,** 7972–7976.
46. Caruso-Neves, C., Meyer-Fernandes, J. R., Saad-Nehme, J., and Lopes, A. G. (1998) *Z. Naturforsch. C* **53,** 911–917.

47. Caruso-Neves, C., Meyer-Fernandes, J. R., Saad-Nehme, J., Proverbio, F., Marín, R., and Lopes, A. G. (1998) *Comp. Biochem. Physiol. B* **119,** 807–811.
48. Sarkis, J. J. F., Guimarães, J. A., and Ribeiro, J. M. (1986) *Biochem. J.* **233,** 885–890.
49. Barbacci, E., Filippini, A., De Cesaris, P., and Ziparo, E. (1996) *Biochem. Biophys. Res. Commun.* **222,** 273–279.
50. Hourani, S. M. O., and Chown, J. A. (1989) *Gen. Pharmacol.* **20,** 413–416.
51. Ziganshin, A. U., Ziganshina, L. E., King, B. F., and Burnstock, G. (1995) *Pflügers Arch.* **429,** 412–418.
52. Lin, S.-H. (1985) *J. Biol. Chem.* **260,** 7850–7856.
53. Beukers, M. W., Pirovano, I. M., Van Weert, A., Kerkhof, C. J. M., Ijzerman, A. P., and Soudijn, W. (1993) *Biochem. Pharmacol.* **46,** 1959–1966.
54. Bencic, D. C., Yates, T. J., and Ingermann, R. L. (1997) *Physiol. Zool.* **70,** 621–630.
55. Steinberg, T. H., and Silverstein, S. C. (1987) *J. Biol. Chem.* **262,** 3118–3122.
56. Chen, B. C., Lee, C.-M., and Lin, W.-W. (1996) *Br. J. Pharmacol.* **119,** 1628–1634.
57. Clifford, E. E., Martin, K. A., Dalal, P., Thomas, R., and Dubyak, G. R. (1997) *Am. J. Physiol.* **273,** C973–C987.
58. Segel, G. B., Ryan, D. H., and Lichtman, M. A. (1985) *J. Cell Physiol.* **124,** 424–432.
59. Dombrowski, K. E., Ke, Y., Thompson, L. F., and Kapp, J. (1995) *J. Immunol.* **154,** 6227–6237.
60. Dombrowski, K. E., Brewer, K. A., Maleckar, J. R., Kirley, T. L., Thomas, J. W., and Kapp, J. (1997) *Arch. Biochem. Biophys.* **340,** 10–18.
61. Dombrowski, K. E., Ke, Y., Brewer, K. A., and Kapp, J. (1998) *Immunol. Rev.* **161,** 111–118.
62. Handa, M., and Guidotti, G. (1996) *Biochem. Biophys. Res. Commun.* **218,** 916–923.
63. Wang, T.-F., and Guidotti, G. (1996) *J. Biol. Chem.* **271,** 9898–9901.
64. Wang, T.-F., Ou, Y., and Guidotti, G. (1998) *J. Biol. Chem.* **273,** 24814–24821.
65. Zimmermann, H. (1999) *Trends Pharmacol. Sci.* **20,** 231–236.
66. Heine, P., Braun, N., Heilbronn, A., and Zimmermann, H. (1999) *Eur. J. Biochem.* **262,** 102–107.
67. Kansas, G. S., Wood, G. S., and Tedder, T. F. (1991) *J. Immunol.* **146,** 2235–2244.
68. Goding, J. W. (2000) *J. Leukoc. Biol.* **67,** 285–311.
69. Morrison, D. C., and Ryan, J. L. (1987) *Annu. Rev. Med.* **38,** 417–432.
70. Lynn, W. A., and Golenbock, D. T. (1992) *Immunol. Today* **13,** 271–276.
71. Kittel, A. (1999) *J. Histochem. Cytochem.* **47,** 393–399.
72. Gillespie, J. P., Kanost, M., and Trenczek, T. (1997) *Annu. Rev. Entomol.* **42,** 611–643.
73. Hoffman, J. A., Kafatos, F. C., Janeway, C. A., Jr., and Ezekowitz, R. A. B. (1999) *Science* **284,** 1313–1318.