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# Glutamate Interacts With VDAC and Modulates Opening of the Mitochondrial Permeability Transition Pore

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The amino acid glutamate, synthesized in the mitochondria, serves multiple functions, including acting as a neurotransmitter and participating in degradative and synthetic pathways. We have previously shown that glutamate modulates the channel activity of bilayer-reconstituted voltage-dependent anion channel (VDAC). In this study, we demonstrate that glutamate also modulates the opening of the mitochondrial permeability transition pore (PTP), of which VDAC is an essential component. Glutamate inhibited PTP opening, as monitored by transient $Ca^{2+}$ accumulation, mitochondrial swelling and accompanying release of cytochrome *c*. Exposure to L-glutamate delayed the onset of PTP opening up to 3-times longer, with an $IC_{50}$ of 0.5 mM. Inhibition of PTP opening by L-glutamate is highly specific, not being mimicked by D-glutamate, L-glutamine, L-aspartate, or L-asparagine. The interaction of L-glutamate with VDAC and its inhibition of VDAC's channel activity and PTP opening suggest that glutamate may also act as an intracellular messenger in the mitochondria-mediated apoptotic pathway.

**KEY WORDS:** Glutamate; mitochondria; permeability transition pore; porin; VDAC.

## INTRODUCTION

The amino acid L-glutamate is considered to be the major mediator of excitatory signaling in the mammalian central nervous system and is probably involved in many aspects of normal brain function including cognition, memory, and learning (for review, see Fonnum, 1984). Glutamate can be generated through several biochemical pathways, including transamination reactions (Nissim, 1999). In the mitochondria, glutamate is predominantly formed from the tricarboxylic acid cycle intermediate $\alpha$-ketoglutarate by the action of glutamate dehydrogenase. The glutamate formed in the mitochondria is transferred to the cytosol. In the mitochondria, however, glutamate serves as a substrate for enzymes including those involved in glutamine synthesis (Hudson and Daniel, 1993) and as a precursor of glutathione (Hammarqvist *et al.*, 1997). Recently, a novel role for glutamate in insulin secretion in $\beta$-cells has been suggested, where glutamate is proposed to assume an intracellular messenger role (Maechler and Wollheim, 1999, 2000; Rubi *et al.*, 2001). The putative function of glutamate as a second messenger has been substantiated in a transgenic mouse model (Shi *et al.*, 2000), however, the suggestion that glutamate acts as a signaling molecule in insulin secretion remains a matter of debate (MacDonald and Fahien, 2000).

We have shown that glutamate, at relatively low concentrations, modulates the channel activity of the bilayer-reconstituted mitochondrial voltage-dependent anion channel (VDAC), a large pore channel located in the mitochondrial outer membrane. Glutamate, at low voltages, converted the highly stable long-lived open state of the VDAC channel to a channel fluctuating between the open and closed states, consequently leading to decreased channel conductance (Gincel *et al.*, 2000, 2002; Shoshan-Barmatz and Gincel, 2003).

VDAC provides passage for nucleotides, $Ca^{2+}$ and other metabolites into and out of the mitochondria (Gincel *et al.*, 2001; Hodge and Colombini, 1997; Rostovtseva and

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Colombini, 1997). VDAC is believed to be a constituent of the mitochondrial mega-channel termed the permeability transition pore (PTP) (Crompton *et al*., 1998; Shoshan-Barmatz and Gincel, 2003; Tsujimoto and Shimizu, 2002; Zoratti and Szabo, 1995). As a consequence of PTP opening, the transmembrane potential collapses, uncoupling of the respiratory chain occurs, and hyperproduction of superoxide anions, release of soluble intermediate proteins (e.g. cytochrome *c*, apoptosis-inducing factor, and procaspase 3), and outflow of matrix $Ca^{2+}$ and glutathione take place (Crompton *et al*., 1998; Smaili *et al*., 2000). Current models suggest that PTP is formed at contact sites between the mitochondrial inner and outer membranes by association of VDAC in the outer membrane with the adenine nucleotide translocator (ANT) in the inner membrane, cyclophilin D (CypD) in the matrix, and possibly other proteins (Crompton *et al*., 1998; Woodfield *et al*., 1998). This proposed PTP structure is consistent with the inhibition and activation of PTP opening by the ANT ligands bongkrekic acid and atractyloside, respectively, and by inhibition by Cyclosporin A (CsA), which binds to cyclophiline D (Crompton *et al*., 1992; Halestrap and Davidson, 1990). Evidence for VDAC being a component of the PTP comes from the regulation of VDAC by antiapoptotic proteins such as Bcl-xL (Vander Heiden *et al*., 2001) and the proapoptotic proteins Bax and Bak (Tsujimoto and Shimizu, 2002), from copurification of VDAC with CypD and ANT (Crompton *et al*., 1998; Kroemer and Reed, 2000), as well as from the inhibition of PTP opening by ruthenium red (RuR) and ruthenium 360 (Ru360), agents that induce closure of bilayer-reconstituted purified VDAC (Gincel *et al*., 2000, 2002). Thus, considering that VDAC is a constituent of the PTP, together with the modulation of VDAC channel activity by glutamate, the effect of glutamate on PTP opening was investigated. In such studies, L-glutamate was found to specifically inhibit mitochondrial $Ca^{2+}$-activated, CsA-sensitive PTP opening.

## EXPERIMENTAL PROCEDURES

### Materials

Asparagines, aspartate, *n*-decane, $CaCl_2$, EDTA, L-glutamate, D-glutamate, glutamine, Hepes, lysine, mannitol, nitrilotriacetic acid, soybean asolectin, sucrose, Trizma base, and Triton X-100 were purchased from Sigma. $^{45}Ca^{2+}$ was purchased from NEN. Alkaline phosphatase-conjugated goat anti-mouse IgG was obtained from Promega. Anticytochrome *c* monoclonal antibodies were obtained from Pharmingen (San Diego, CA).

### Rat Liver Mitochondria Isolation

Rat liver mitochondria were isolated from Sprague-Dawley rats as described previously (Gincel *et al*., 2001). Briefly the livers of anesthetized rats were washed with sucrose buffer (0.25 M sucrose, 0.1 mM EDTA, 10 mM Hepes, pH 7.2, and Sigma protease inhibitor cocktail) and homogenized with a teflon glass motor homogenization in the same solution at ratio of 4:1 (w/v) at 1000 rpm. The homogenate was diluted 1:1 with sucrose buffer and centrifuged at 1000*g* for 5 min. The resulting suspension was centrifuged again at 1000*g* for 5 min and the supernatant was further centrifuged at 12,000*g* for 10 min. The obtained pellet was resuspended in sucrose buffer (without protease inhibitors and EDTA) and recentrifuged at 12,000*g* for 10 min. The final mitochondrial pellet was resuspended in sucrose buffer, stored at 4°C, and used within 4–6 h. The specific activity of succinate-cytochrome *c* oxido-reductase activity (Johnson and Lardy, 1967), before (0) and after osmotic shock ($127.1 \pm 0.5$ nmol/mg ($n = 15$) were used to follow the intactness of the mitochondria. In most experiments, the intactness of the mitochondria was about 99%. Protein concentration was determined according to Lowry *et al.* (1951).

### Mitochondrial $Ca^{2+}$ Accumulation

$Ca^{2+}$ accumulation by freshly isolated rat liver mitochondria (0.5 mg/mL) was assayed for 1–20 min at 30°C in the presence of 220 mM mannitol, 70 mM sucrose, 0.5 mM nitrilotriacetic acid, 120 $\mu$M $CaCl_2$ (containing $^{45}Ca^{2+}$ $3 \times 10^4$ cpm/nmol) (free $[Ca^{2+}] = 33.6$ $\mu$M), 5 succinate, 0.05–0.1 mM Pi, and 15 mM Tris/HCl, pH 7.2. $Ca^{2+}$ accumulation was terminated by rapid filtration followed by washing with 5 mL of 0.15 M KCl. The free $Ca^{2+}$ concentration was calculated with WinMAXC 2.05 software (Bers *et al*., 1994), using a Log stability constant for nitrilotriacetic acid and a $Ca^{2+}$ complex value of 6.5 (Dawson *et al*., 1986). The maximal amount of $Ca^{2+}$ accumulated in mitochondria was about 550 nmol/mg protein (eight different mitochondrial preparations).

### Mitochondrial Swelling

$Ca^{2+}$-induced mitochondria large amplitude swelling was assayed in freshly-prepared mitochondria under the same conditions as for $Ca^{2+}$ accumulation, except that the temperature was 24°C. Swelling was also assayed under the conditions described below for the

assay of cytochrome *c* release. Swelling was initiated upon addition of $Ca^{2+}$ (0.12 mM) to the sample cuvette, with the absorbance changes at 520 nm monitored every 15–20 s and measured with an Ultraspec 2100 spectrophotometer.

### Release of Cytochrome *c*

Release of cytochrome *c* was assayed under the conditions were PTP is opened or closed, as reflected in mitochondrial swelling preformed according to Pastorino *et al*. (2002) in KCl medium containing 150 mM KCl, 25 mM $NaHCO_3$, 1 mM $MgCl_2$,3 mM $KH_2PO_4$, 20 mM Hepes, pH 7.4, with succinate (5 mM) added as the respiratory substrate. Mitochondria, incubated under different conditions, were centrifuged at 20,000*g* and the membranal and soluble fractions were subjecting to SDS-PAGE and immunoblot analysis using monoclonal anticytochrome *c* antibodies.

### VDAC Purification

VDAC was purified from mitochondria as described previously using Triton X-100 and reactive red-agarose (Gincel *et al*., 2001).

### Single Channel Recording and Analysis

Reconstitution of purified VDAC into planar lipid bilayer (PLB), single channel current recording, and data analyses were carried out as previously described (Gincel *et al*., 2001). Briefly, PLB were prepared from soybean asolactin dissolved in *n*-decane (50 mg/mL). Purified VDAC (about 1 ng) was added to the chamber defined as *cis*. Following insertion of a single or few channels into the PLB, the excess protein was removed by exchanging the solution in the *cis* chamber with fresh buffer. Currents were measured with respect to the *trans* side of the membrane (ground) and were recorded under voltage-clamp conditions using a Bilayer Clamp BC-525B amplifier (Warner Instrument). The currents were low-pass filtered at 1 kHz using a Bessal filter (Frequency Devices 902) and digitized on-line using a Digidata 1200 interface board and pCLAMP 6 software (Axon Instruments).

## RESULTS

### L-Glutamate Modulates VDAC Channel Activity

To demonstrate the interaction of glutamate with VDAC, D- and L-glutamate were added to purified VDAC reconstituted into a planar lipid bilayer. Whereas, in the absence of L-glutamate, VDAC is stable in a long-lived, fully opened state at low voltage (10 mV) (Fig. 1, control), addition of L-glutamate to the same channel induced channel fluctuations between the fully open and subconducting states (Fig. 1, L-glutamate lower trace). The stereoisomer D-glutamate had no effect on channel activity, with the channel remaining in its open state for over 30 min (Fig. 1, D-glutamate), indicating the specificity of the L-glutamate binding site.

### Glutamate Modulation of PTP Opening

Since VDAC is believed to be a constituent of the PTP (Crompton *et al*., 1998; Shoshan-Barmatz and Gincel, 2003; Tsujimoto and Shimizu, 2002; Zoratti and Szabo, 1995), interaction of L-glutamate with VDAC and modulation of its channel activity would likely be reflected in PTP opening. Thus, the effect of glutamate on PTP opening, as induced by mitochondria $Ca^{2+}$-overload or by atractyloside was studied.

PTP opening, as monitored by mitochondrial swelling, was modified by L-glutamate (Fig. 2). At relatively low concentrations (0.5 or 5 mM), glutamate lengthened the time needed for the onset of $Ca^{2+}$-dependent PTP opening. This effect is different from the effects of EGTA and CsA, which completely prevented PTP opening (Fig. 2(A)). In order to rule out the possibility that glutamate is influencing the onset of mitochondrial swelling by acting as a substrate, we used succinate as an electron source in higher concentrations in both mitochondrial swelling and $Ca^{2+}$ accumulation, and this had no effect on glutamate modification of PTP. Furthermore, the level of electron transport from succinate to cytochrome *c* was the same in the absence and the presence of glutamate. Similar results showing that in the presence of succinate, glutamate had no effect on oxygen uptake were reported (Wieckowski and Wojtczak, 1997).

To estimate the affinity of the L-glutamate binding sites involved in PTP modulation, the time required for half-maximal mitochondrial swelling was determined as a function of L-glutamate concentration (Fig. 2(B)). The time required for half-maximal swelling increased by up to threefold with increasing glutamate concentration, with half-maximal inhibition ($IC_{50}$) of PTP opening occurring at 1.1 mM (Fig. 2(B)). The half-maximal decrease in VDAC conductance was obtained at the similar concentration of 3.8 mM (Gincel *et al*., 2000).

L-Glutamate was also found to affect PTP opening as monitored by transient mitochondrial $Ca^{2+}$ accumulation,

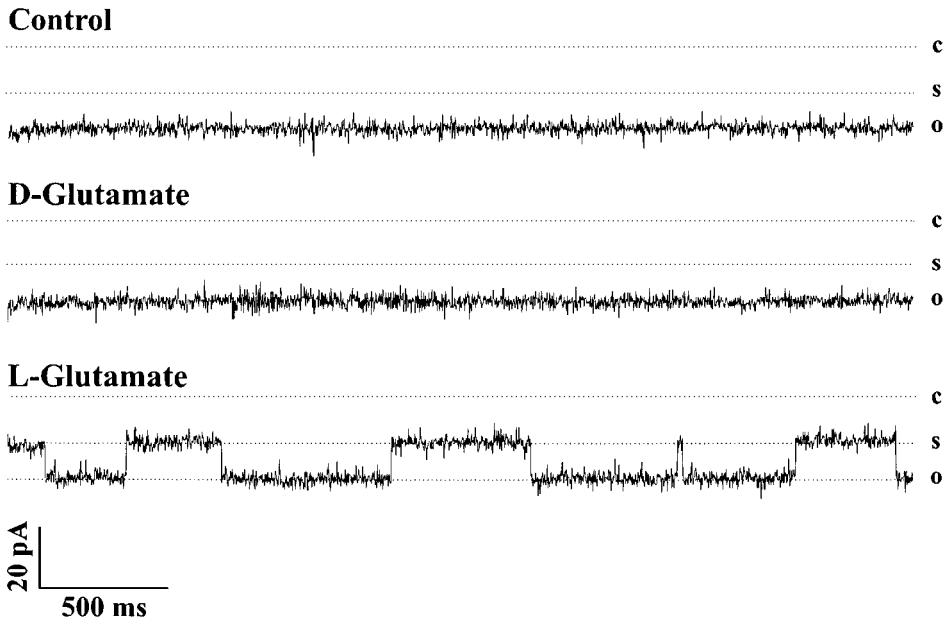


**Fig. 1.** L-Glutamate but not D-glutamate modulates VDAC channel activity. Purified VDAC was reconstituted into PLB and channel activity was analyzed as described in Experimental Procedures section. The currents passing though single VDAC molecule in response to a voltage step from 0 to −10 mV were recorded before (upper trace) and after the addition 10 mM of D-glutamate (middle trace) or L-glutamate (lower trace). c indicates closed channel ($I = 0$) and o and s indicate open and substates of VDAC channel.

where the mitochondrial $Ca^{2+}$ content reaching a maximal level and then rapidly decreased to less then 1–2% of its maximal value (Fig. 2(C)). If L-glutamate was added after $Ca^{2+}$ accumulation had reached the maximal level, yet before PTP opening, glutamate delayed PTP opening (Fig. 2(C)). Mitochondrial PTP opening is also accompanied by cytochrome *c* release. $Ca^{2+}$-activated, CsA-sensitive cytochrome *c* release from mitochondria was inhibited by L-glutamate, as analyzed by western blotting (Fig. 2(D)). Upon PTP opening, cytochrome *c* was released into the supernatant. This release was diminished by 5 $\mu$M CsA, a concentration that completely prevented PTP opening (see Fig. 2(A)). Under the conditions employed, the release of cytochrome *c* from mitochondria was inhibited by L-glutamate (62%).

### Glutamate Modulation of PTP Is Specific

To investigate whether the effect of L-glutamate on PTP opening was specific and not due to a possible direct interaction with the $Ca^{2+}$ required for PTP activation, the effects of other structurally-related and unrelated amino acids on mitochondrial swelling were tested (Fig. 3). Modulation of PTP opening was found to be specific for L-glutamate and was not observed with L-aspartate, L-asparigine, L-lysine, or L-glutamine (Fig. 3(A)). The specificity of the L-glutamate binding site in PTP was further demonstrated by comparing the effects of the stereoisomer of L-glutamate, i.e., D-glutamate, on mitochondrial swelling. As observed with the modulation of VDAC channel activity (Fig. 1), D-glutamate had no effect on PTP opening, as reflected by an absence of mitochondrial swelling (Fig. 3(B)). Moreover, release of cytochrome *c* from the PTP-activated mitochondria was inhibited by L-glutamate, but not by D-glutamate (Fig. 3(B), inset). These results rule out the possibility that the L-glutamate-mediated effect is due to a $Ca^{2+}$-chelating ability of the amino acid.

### Glutamate Modulation of PTP Is due to Its Interaction With VDAC

Atractyloside is an ANT ligand previously shown to interact with PTP and stabilize the open state (Beutner *et al*., 1998). L-Glutamate inhibited atractyloside-induced PTP opening via the nonmitochondrial $Ca^{2+}$-overload pathway (Fig. 4(A)). This finding supports the above

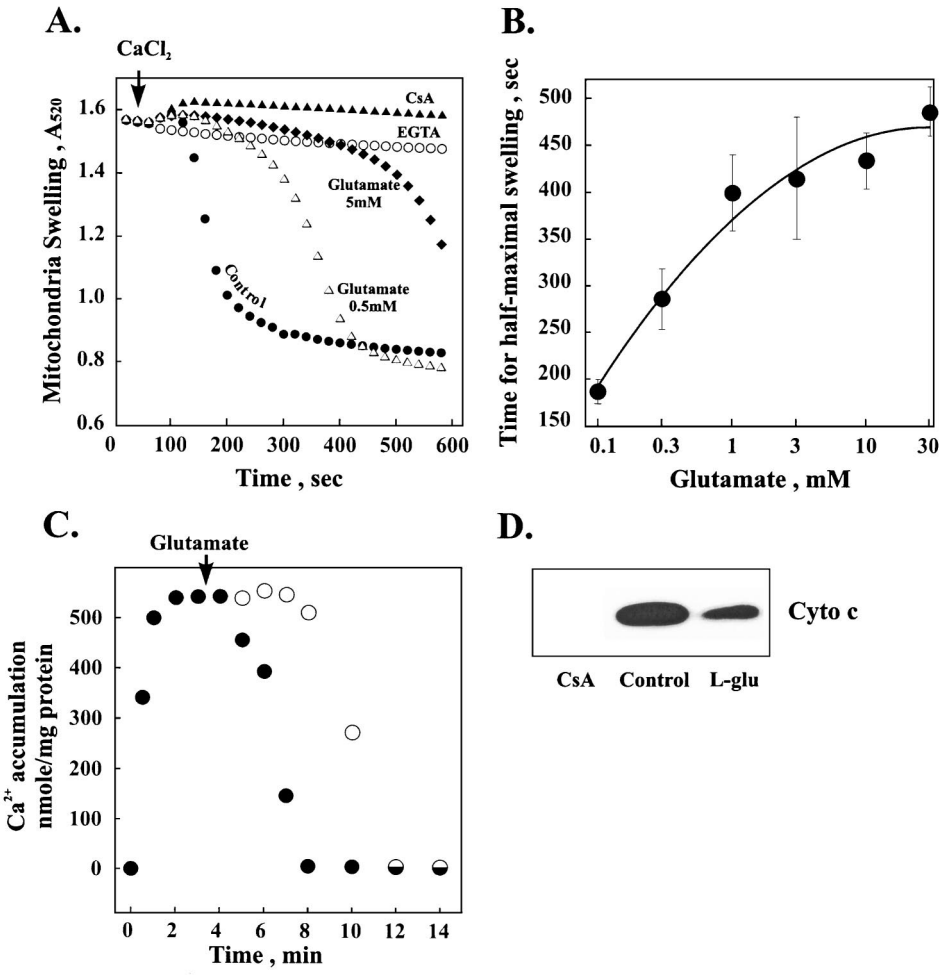


**Fig. 2.** Modulation of PTP opening by glutamate. (A) CsA-sensitive mitochondrial swelling, followed by decrease in absorbance at 520 nm, was assayed in the absence and the presence of cyclosporine A (CsA, 1 $\mu$M), EGTA (1 mM), or in the presence of the indicated concentration of glutamate. (B) The time required for half-maximal swelling in the absence and the presence of different concentrations of glutamate was measured and is presented as a function of glutamate concentration. (C) Succinate-supported $^{45}Ca^{2+}$ accumulation by rat liver mitochondria (0.5 mg/mL) was assayed for the indicated times. After $Ca^{2+}$ accumulation had reached a maximal level (indicated by an arrow), glutamate (3 mM) was added and mitochondrial $Ca^{2+}$ content was measured at the indicated times, for control (○) and glutamate containing sample (●). (D) Mitochondria in a KCl buffer were incubated for 8 min without or with 5 mM L-glutamate or 5 $\mu$M cyclosporine D prior to their centrifugation at 20,000*g*. The obtained supernatants were then subjected to SDS-PAGE and cytochrome *c* levels were assayed using monoclonal anticytochrome *c* antibodies (Pharmingen, San Diego, CA) and anti-mouse HRP-conjugated antibodies as the secondary antibody.

conclusion that the L-glutamate-mediated effect is due to its direct interaction with VDAC and not through interactions with $Ca^{2+}$ or $Ca^{2+}$-transport systems. Moreover, the results indicate that L-glutamate was able to inhibit PTP opening when ANT was stabilized in its open conformation by atractyloside, further suggesting an interaction of L-glutamate with VDAC or cyclophyline D.

Finally, the results in Fig. 4(B) indicate that L-glutamate had no effect on mitochondrial swelling when the intactness of the outer mitochondrial membrane, the site of VDAC, was initially damaged by osmotic shock or freeze-thaw treatment.

## DISCUSSION

In a previous study, we showed that L-glutamate modified bilayer-reconstituted VDAC channel activity

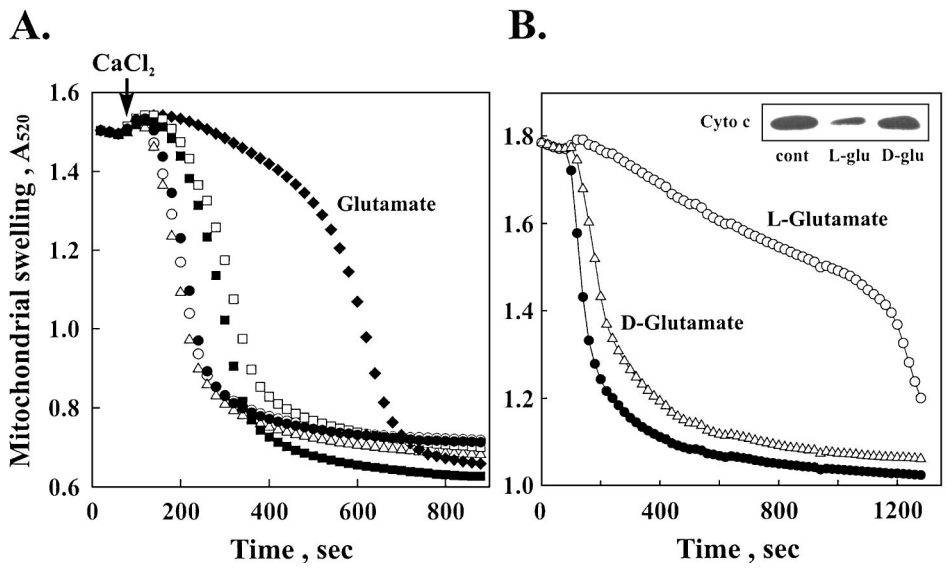


**Fig. 3.** Glutamate modulation of PTP is specific and not due to interaction with $Ca^{2+}$. (A) Mitochondrial swelling was measured as in Fig. 2, except that the indicated amino acid (5 mM) was present during the swelling assay. L-Glutamate (♦), L-aspartate (○), L-lysine (△), glutamine (□), asparagine (■), and control (●). (B) Mitochondrial swelling was induced in the absence (●) or the presence of L-glutamate (○) or D-glutamate (△). Inset shows the amount of cytochrome *c* released (see Fig. 2) upon PTP induction in the absence or presence (cont) of 5 mM L-glutamate (L-glu) or 5 mM D-glutamate (D-glu).

and induced rapid fluctuation between open and subconducting states of the channel (Gincel *et al*., 2000). L-Glutamate's effect on VDAC activity is specific, since it was observed at high ionic strength (1 M NaCl) and could not mimicked by D-glutamate (Fig. 1). These results suggest that VDAC possesses a specific L-glutamate binding site(s) that modulates VDAC activity.

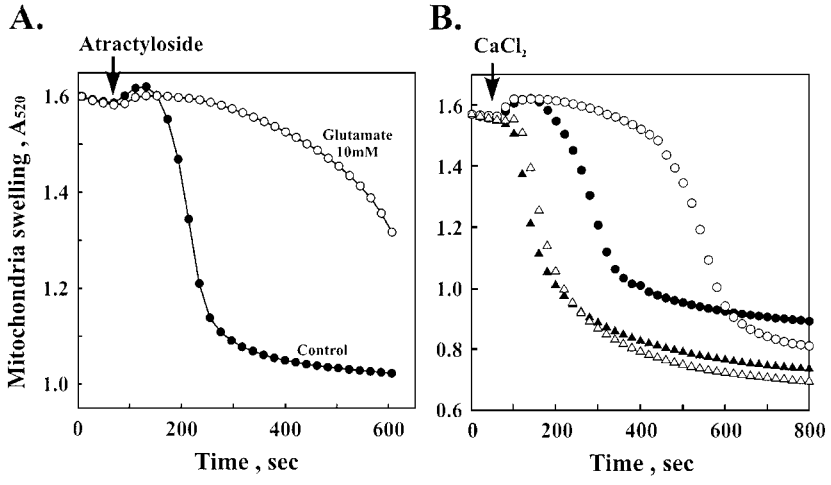


**Fig. 4.** Glutamate inhibits atractyloside-induced PTP opening and only in intact OMM. (A) Swelling initiated by atractyloside (0.2 $\mu$M) in the presence of low concentrations of $Ca^{2+}$ (20 $\mu$M) is inhibited by glutamate. (B) L-Glutamate inhibition of mitochondrial swelling was assayed in mitochondria with intact (○, ●) and nonintact (△, ▲) OMM, as obtained by repeated freeze-thaw cycles. L-Glutamate inhibited swelling only in mitochondria containing the outer membrane (○) but not in nonintact outer mitochondrial membrane (△). Arrows indicate the time of atractyloside or $CaCl_2$ addition.

In this study, we show that L-glutamate not only modified VDAC's channel activity, but that it also inhibited PTP opening. L-Glutamate, but not other amino acids tested, including D-glutamate, affected PTP opening. The similar specificity of the L-glutamate binding sites in VDAC and PTP, i.e., with binding being specific to L-glutamate but not to D-glutamate, L-aspartate, or other amino acids (Figs. 1 and 3), may suggest that the same binding site is involved in the glutamate-mediated effects on VDAC conductance and on PTP opening. This suggestion also supports the hypothesis that VDAC is a constituent of the PTP (Crompton *et al*., 1998; Tsujimoto and Shimizu, 2002; Zoratti and Szabo, 1995), and its regulation of PTP opening (Shoshan-Barmatz and Gincel, 2003). Moreover, the observation that the effect of L-glutamate on PTP opening is detected only in intact OMM suggests that glutamate, by interacting with VDAC, modulates the permeability of intact, but not of nonintact OMM, further supports the function of VDAC in controlling OMM permeability.

Mitochondrial functions were shown to be regulated by glutamate and other respiratory substrates that profoundly affect mitochondrial responses to manipulations of pathophysiological relevance, such as PTP opening (Leverve and Fontaine, 2001). However, glutamate-interacting targets have not yet been identified. The results presented here point to VDAC as the glutamate-interacting site, and thereby, modulation of mitochondrial activities is mediated by VDAC activity.

Glutamate serves several catabolic and anabolic functions in the cell cycle, and as a neurotransmitter. Glutamate was recently allocated a new role, namely that of an intracellular messenger or cofactor in insulin secretion (Maechler and Wollheim, 1999, 2000; Rubi *et al*., 2001). Extracellular glutamate also interacts with its receptors in postsynaptic spines to induce neuronal apoptosis through mitochondrial $Ca^{2+}$-overload (for review, see Mattson, 2000). Thus, the control of glutamate concentration in the cell and in the extracellular matrix is very important. Several transport systems regulate glutamate localization and concentration in the cell (Heath and Shaw, 2002). In the central nervous system, glutamate exists within metabolic and neurotransmitter pools, where the cellular metabolic pool constitutes approximately 80% of the stored glutamate (Heath and Shaw, 2002). In such cells, glutamate is synthesized in the mitochondria from where it is transported to the cytosol. However, to be transported into the cytosol, glutamate must cross not only the inner mitochondria membrane, but also the OMM. Since the only known transporter of ions and metabolites across the OMM is VDAC, glutamate concentration in the cytosol is primary controlled by the open state of VDAC. Our results suggest that glutamate itself could control its own concentration in the cytosol via direct interaction with VDAC. Indeed, as previously demonstrated, glutamate decreased VDAC conductance to small ions and completely prevented its own transport (Gincel *et al.*, 2000). This is directly relevant given the involvement of glutamate in various disease states and in glutamate-mediated toxicity, with decreased glutamate levels, altered expression, and function of glial glutamate transporter and other factors being affected in such states (see Atlante *et al.*, 2001; Heath and Shaw, 2002).

L-Glutamate binding sites have been characterized and localized in only few proteins. It has been postulated that between 4 and 12 asparagine residues in the glutamate receptors participate in glutamate binding (Standley and Baudry, 2000). Upon analysis of the amino acid sequence presented in the proposed topology model for VDAC (Song *et al*., 1998), one can identify two possible glutamate binding sites each containing nine asparagines, that might form a binding site in a three-dimensional structure. One site faces the cytoplasm while the other faces the mitochondria matrix.

Further definition of the VDAC glutamate binding site and the molecular mechanism underlying the role of glutamate as a second messenger in a pathway so significant to cell life such as apoptosis should help to define the putative implications of glutamate in other processes.

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