Naunyn-Schmiedeberg's Arch Pharmacol (2011) 384:59–70
DOI 10.1007/s00210-011-0630-1

ORIGINAL ARTICLE

Idebenone inhibition of glutamate release from rat cerebral cortex nerve endings by suppression of voltage-dependent calcium influx and protein kinase A

Yi Chang · Yu-Wan Lin · Su-Jane Wang

Received: 9 January 2011 / Accepted: 25 March 2011 / Published online: 4 May 2011
© Springer-Verlag 2011

Abstract The present study was aimed at investigating the effect and the possible mechanism of idebenone on endogenous glutamate release in nerve terminals of rat cerebral cortex (synaptosomes). Idebenone inhibited the release of glutamate that was evoked by exposing synaptosomes to the K^+ channel blocker 4-aminopyridine (4-AP), and this phenomenon was concentration dependent. Inhibition of glutamate release by idebenone was prevented by chelating extracellular Ca^{2+}, or by the vesicular transporter inhibitor bafilomycin A1, but was insensitive to DL-threo-beta-benzyl-oxyaspartate, a glutamate transporter inhibitor. Idebenone decreased the depolarization-induced increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_C$),whereas it did not alter the resting synaptosomal membrane potential or 4-AP-mediated depolarization. The inhibitory effect of idebenone on evoked glutamate release was prevented by blocking the $Ca_v2.2$ (N-type) and $Ca_v2.1$ (P/Q-type) channels, but not by blocking intracellular Ca^{2+} release or Na^+/Ca^{2+} exchange. Furthermore, the idebenone effect on 4-AP-evoked Ca^{2+} influx and glutamate release was completely abolished by the protein kinase A (PKA) inhibitors, H89 and KT5720. On the basis of these results, it was concluded that idebenone inhibits glutamate release from rat cortical synaptosomes and this effect is linked to a decrease in $[Ca^{2+}]_C$ contributed by Ca^{2+} entry through presynaptic voltage-dependent Ca^{2+} channels and to the suppression of PKA signaling cascade.

Keywords Idebenone · Glutamate release · $Ca_v2.2$ (N-type) and $Ca_v2.1$ (P/Q-type) channels · Protein kinase A · Synaptosomes · Cerebral cortex

Y. Chang
Department of Anesthesiology,
Shin Kong Wu Ho-Su Memorial Hospital,
95 Wen-Chang Road,
Taipei 111, Taiwan

Y. Chang
School of Medicine,
Fu jen Catholic University,
No. 510, Zhongzheng Rd., Xinzhuang Dist.,
New Taipei City 24205, Taiwan

Y.-W. Lin
Graduate Institute of Basic Medicine, Fu Jen Catholic University,
No. 510, Zhongzheng Rd., Xinzhuang Dist.,
New Taipei City 24205, Taiwan

S.-J. Wang (✉)
Graduate Institute of Basic Medicine, and School of Medicine,
Fu Jen Catholic University,
No. 510, Zhongzheng Rd., Xinzhuang Dist.,
New Taipei City 24205, Taiwan
e-mail: med0003@mail.fju.edu.tw

Introduction

In the brain, glutamate is a major excitatory neurotransmitter that plays an important role in many functions such as synaptic plasticity, learning, and memory (Greenamyre and Porter 1994; Danbolt 2001). Besides its physiological role, glutamate is also a potent neurotoxin and high levels in the synaptic cleft may induce an increase in intracellular Ca^{2+} levels, which in turn triggers a cascade of cellular responses, including enhanced oxygen-free radical production, disturbed mitochondrial function, and protease activation, that ultimately kill the neurons (Coyle and Puttfarcken 1993; Schinder et al. 1996). This process has been proposed to be involved in a number of neuropathological conditions, ranging from acute insults such as stroke, epileptic seizures, traumatic brain, and spinal cord injury, to chronic neurodegenerative disorders such as Alzheimer's disease, Parkinson's

disease, and amyotrophic lateral sclerosis (Obrenovitch and Urenjak 1997; Meldrum 2000; Raiteri et al. 2004).As a consequence, affecting glutamate neurotransmission such as inhibiting glutamate release at nerve terminals may be one of the targets for neuroprotective actions.

Idebenone, a centrally potent antioxidant (Suno and Nagaoka 1984), has been found to offer neural protective actions in various experimental studies in vitro and in vivo. It has been demonstrated, for example, that idebenone attenuates glutamate- or β-amyloid-induced neurotoxicity (Miyamoto et al. 1989; Miyamoto and Coyle 1990; Bruno et al. 1994; Hirai et al. 1998; Pereira et al. 1999), protects against ischemia-induced brain damage (Nagaoka et al. 1989), as well as ameliorates learning and memory deficits in rats caused by cerebral ischemia, cerebral embolization, β-amyloid, and aging (Yamazaki et al. 1989; Pelleymounter and Cullen 1993; Yamada et al. 1999). Furthermore, several clinical studies have showed that idebenone is effective in various forms of impairment of brain functions, especially those due to cerebrovascular and neurodegenerative disorders (Zs.-Nagy 1990; Nappi et al. 1992; Senin et al. 1992; Weyer et al. 1997). The mechanisms responsible for the neuroprotective effects of idebenone are, however, not fully clarified, although it has been reported that this beneficial effect is associated with scavenging oxygen-free radicals, increasing cerebral blood flow, and improving brain metabolism (Miyamoto et al. 1989; Nagai et al. 1989; Nagaoka et al. 1989; Cardoso et al. 1998).

Since excessive release of glutamate is a critical factor in the neuropathology of acute and chronic brain disorders, it is possible that the neural protective effect of idebenone is associated with a decrease in glutamate release. However, there are no previous studies about the effect of idebenone on glutamate release in central neurons. As a result, this study used isolated nerve terminals (synaptosomes) purified from the rat cerebral cortex to investigate the effect of idebenone on glutamate release and to characterize the underlying molecular mechanisms. The isolated presynaptic terminal represents a model system for investigating directly the molecular mechanisms underlying presynaptic phenomena. Specifically, this preparation is capable of accumulating, storing, and releasing neurotransmitters, and is devoid of functional glial and nerve cell body elements that might obfuscate interpretation because of modulatory loci at non-neuronal, postsynaptic, or network levels (Dunkley et al. 1986). The experiments were performed with synaptosomes by monitoring the effects of idebenone on the levels of endogenous released glutamate, the synaptosomal plasma membrane potential, and the downstream activation of voltage-dependent Ca^{2+} channels (VDCCs). In addition, in view of the demonstrated role of various kinases, including protein kinase C (PKC) and protein kinase A (PKA), in presynaptic modulation (Herrero and Sanchez-Prieto 1996; Wang et al. 2002; Millan et al. 2003; Wang and Sihra 2003), this study also examined whether protein kinase signaling pathways were involved in the regulation of idebenone on glutamate release.

Materials and methods

Animals

Adult male Sprague–Dawley rats (250–300 g) were employed in these studies. All animal procedures were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and were approved by the Fu Jen Institutional Animal Care and Utilization Committee.

Drugs

3′,3′-Dipropylthiadicarbocyanine iodide [$DiSC_3(5)$] and fura-2-acetoxymethyl ester (Fura-2-AM) were obtained from Invitrogen (Carlsbad, CA, USA). Idebenone, bafilomycin A1, ω-conotoxin (CgTX)MVIIC, dantrolene, 2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo-benzodiazocine-10-carboxylic acid-hexyl ester (KT5720), DL-threo-beta-benzyl-oxyaspartate (DL-TBOA), N-[2-(p-bromocinnamylamino)-ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89), and bisindolylmaleimide I (GF109203X) were obtained from Tocris Cookson (Bristol, UK). Glutamate dehydrogenase, nicotinamide adenine dinucleotide phosphate ($NADP^+$), ethylene glycol tetraacetic acid (EGTA), Ro318220, and all other reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

Synaptosomal preparation

Synaptosomes were prepared as described previously (Nicholls and Sihra 1986; Wang and Sihra 2004). Briefly, the cerebral cortex from 2-month-old male Sprague–Dawley rats was isolated and homogenized in a medium that contained 320 mM sucrose, pH 7.4. The homogenate was spun for 2 min at 3,000×*g* (5,000 rpm in a JA 25.5 rotor; Beckman Coulter, Inc., USA) at 4°C, and the supernatant was spun again at 14,500×*g* (11,000 rpm in a JA 25.5 rotor) for 12 min. The pellet was gently resuspended in 8 ml of 320 mM sucrose, pH 7.4. Two milliliters of this synaptosomal suspension was added to 3 ml Percoll discontinuous gradients that contained 320 mM sucrose, 1 mM EDTA, 0.25 mM DL-dithiothreitol, and 3, 10, and 23% Percoll, pH 7.4. The gradients were centrifuged at 32,500×*g* (16,500 rpm in a JA

20.5 rotor) for 7 min at 4°C. Synaptosomes placed between the 10% and 23% Percoll bands were collected and diluted in a final volume of 30 ml of HEPES buffer medium (HBM) that consisted of 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2{\cdot}6H_2O$, 1.2 mM Na_2HPO_4, 10 mM glucose, and 10 mM HEPES (pH 7.4), before centrifugation at 27,000×*g* (15,000 rpm in a JA 25.5) for 10 min. The pellets thus formed were resuspended in 3 ml of HBM, and the protein content was determined using a Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA), based on the method of Bradford (1976), with BSA as a standard. 0.5 mg of synaptosomal suspension was diluted in 10 ml of HBM and spun at 3,000×*g* (5,000 rpm in a JA 20.1 rotor) for 10 min. The supernatants were discarded, and the synaptosomal pellets were stored on ice and used within 4–6 h.

Glutamate release assay

Glutamate release from purified cerebrocortical synaptosomes was monitored online, with an assay that employed exogenous glutamate dehydrogenase (GDH) and $NADP^+$ to couple the oxidative deamination of the released glutamate to the generation of NADPH detected fluorometrically. This method allows the detection of submicromolar concentrations of glutamate without interference from other amino acids (McMahon and Nicholls 1991). Because an inherent delay is introduced by the time required for the reaction to occur, fluorometric traces do not reflect the actual glutamate concentration. Initial rates of glutamate release associated with depolarization may be underestimated as corrections for this delay were not incorporated (Rawls et al. 1999). Actually, this online enzyme-linked fluorescent detection from synaptosomes has long been considered an approach particularly appropriate to investigate the presynaptic regulation of neurotransmitter release by drugs (Nicholls and Sihra 1986; Yang and Wang 2009).In brief, synaptosomes (0.5 mg/ml) were resuspended in HBM that contained 16 μM BSA and incubated in an LS-50B spectrofluorimeter (Perkin-Elmer Life Sciences) at 37°C with stirring. $NADP^+$ (2 mM), GDH (50 units/ml), and $CaCl_2$ (1 mM) were added after 3 min. In experiments that investigated Ca^{2+}-independent efflux of glutamate, EGTA (200 μM) was added in place of $CaCl_2$. Other additions before depolarization were made as described in the figure legends. After a further 10 min of incubation, 4-aminopyridine (4-AP) (1 mM), or KCl (15 mM) was added to stimulate glutamate release. Glutamate release was monitored by measuring the increase of fluorescence (excitation and emission wavelengths of 340 and 460 nm, respectively) caused by NADPH being produced by oxidative deamination of released glutamate by GDH. A standard of exogenous glutamate (5 nmol) was added at the end of each experiment, and the fluorescence response used to calculate released glutamate, expressed as nanomoles of glutamate per milligram of synaptosomal protein (nanomoles per milligram). Release values quoted in the text and expressed in bar graphs are levels attained at steady-state after 5 min of depolarization (nanomoles per milligram per 5 min). Data were accumulated at 2-s intervals. Cumulative data were analyzed in Lotus 1-2-3 and MicroCal Origin. Estimation of the IC_{50} was based on a one-site model $[\text{Inhibition} = (\text{Inhibition}_{MAX} \times [\text{idebenone}] / (IC_{50} + [\text{idebenone}]))]$ using the nonlinear curve-fitting function in MicroCal Origin. Statistical analysis was performed by two-tailed Student's *t* test or one-way ANOVA, followed by post hoc two-tailed *t* test.

Cytosolic free Ca^{2+} concentration measurement

Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_C$) was measured using the Ca^{2+} indicator Fura-2-AM. Synaptosomes (0.5 mg/ml) were resuspended in 1 ml of HBM containing $CaCl_2$ (0.1 mM) and loaded with Fura-2-AM (5 μM) for 30 min at 37°C in a stirred test tube. Synaptosomes were washed with HBM by centrifugation, resuspended in 2 ml of HBM, and placed in an LS-50B spectrofluorimeter (Perkin-Elmer Life Sciences) at 37°C with stirring in the presence of $CaCl_2$ (1 mM).Synaptosomes were incubated for 10 min in the presence of idebenone (50 μM) prior to depolarization with 4-AP (1 mM). Fluorescence data were accumulated at excitation wavelengths of 340 and 380 nm (emission wavelength, 505 nm) at 7.5-s intervals. Calibration procedures were performed as described previously (Sihra et al. 1992), using 0.1% SDS to obtain the maximal fluorescence with Fura-2 saturation with Ca^{2+}, followed by 10 mM EGTA (Tris-buffered) to obtain minimum fluorescence in the absence of any Fura-2/Ca^{2+} complex. $[Ca^{2+}]_C$ was calculated using equations described previously (Grynkiewicz et al. 1985). Cumulative data were analyzed in Lotus 1-2-3 and MicroCal Origin. Changes in $[Ca^{2+}]_C$, 5 min after the addition of 4-AP in the presence of idebenone, were calculated. Statistical analysis was performed by two-tailed Student's *t* test or one-way ANOVA, followed by post hoc two-tailed *t* test.

Synaptosomal plasma membrane potential measurement

The synaptosomal membrane potential can be monitored by positively charged, membrane-potential-sensitive carbocyanine dyes such as $DiSC_3(5)$. $DiSC_3(5)$ is a positively charged carbocyanine that accumulates in polarized synaptosomes that are negatively charged on the inside. At high concentrations, the dye molecules accumulate and the fluorescence is quenched. Upon depolarization, the dye moves out and hence the fluorescence increases (Akerman et al. 1987). Synaptosomes were preincubated and resuspended as described for the glutamate release experiments.

After 3-min incubation at 37°C in a stirred test tube, 5 μM $DiSC_3(5)$ was added and allowed to equilibrate before the addition of $CaCl_2$ (1 mM) after 4-min incubation. 4-AP (1 mM) was added to depolarize the synaptosomes at 10 min, and $DiSC_3(5)$ fluorescence was monitored at excitation and emission wavelengths of 646 and 674 nm, respectively. Cumulative data were analyzed in Lotus 1-2-3 and MicroCal Origin, and results are expressed in fluorescence units. Statistical analysis was performed by two-tailed Student's *t* test or one-way ANOVA, followed by post hoc two-tailed *t* test.

Results

Effect of idebenone on 4-AP-evoked glutamate release from rat cerebrocortical nerve terminals

To investigate the influence of idebenone on glutamate release, isolated nerve terminals were depolarized with the K^+-channel blocker 4-AP. 4-AP destabilizes the membrane potential and is thought to cause repetitive spontaneous Na^+-channel-dependent depolarization that closely approximates the in vivo depolarization of the synaptic terminal, which leads to the activation of VDCCs and neurotransmitter release (Nicholls 1998). In synaptosomes incubated in the presence of 1 mM $CaCl_2$, 4-AP evoked a glutamate release of 7.2±0.1 nmol/mg/5 min. Application of idebenone (50 μM) produced an inhibition of 4-AP-evoked glutamate release to 3.7±0.1 nmol/mg/5 min (n=6; P< 0.01), without altering the basal release of glutamate (Fig. 1A). The idebenone-induced inhibition of 4-AP-evoked glutamate release was concentration dependent (Fig. 1B). A robust inhibition of evoked glutamate release was seen with 50 μM idebenone. Because this effect was on the linear part of the concentration–response curve, this concentration was used in subsequent experiments to evaluate the mechanisms that underlie the ability of idebenone to reduce glutamate release. The IC_{50} value for the idebenone-mediated inhibition of 4-AP-evoked glutamate release could not be determined precisely since the maximum extent of inhibition could not be determined.

Effect of Ca^{2+} chelation, DL-TBOA, or bafilomycin A1 on inhibition of 4-AP-evoked glutamate release by idebenone

To test whether the inhibition of 4-AP-evoked glutamate release by idebenone is mediated by an exocytotic system, the Ca^{2+} dependence of the release and the involvement of exocytosis were examined. First, the Ca^{2+}-independent glutamate efflux was measured by depolarizing the synaptosomes with 1 mM 4-AP in extracellular-Ca^{2+}-free solution containing EGTA (300 μM). Under these con-

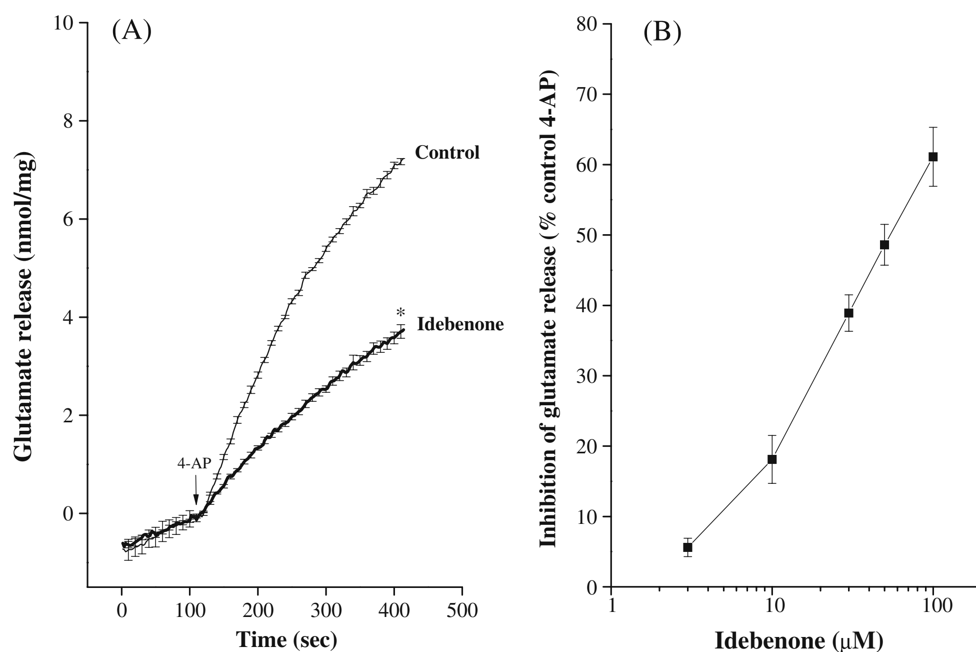

Fig. 1 Idebenone inhibits 4-AP-evoked glutamate release from rat cerebrocortical nerve terminals in a concentration-dependent manner. Synaptosomes were resuspended in incubation medium at a final protein concentration of 0.5 mg/ml and incubated for 3 min before the addition of 1 mM $CaCl_2$. 4-AP (1 mM) was added after 10 min further to effect depolarization (*arrow*). **A** Total glutamate release was measured under control conditions or in the presence of 50 μM idebenone added 10 min prior to the addition of 4-AP. **B** Dose–response curve for idebenone inhibition of 4-AP-evoked glutamate release, showing percentage inhibition compared with controls. Results are the mean±SEM values of independent experiments, using synaptosomal preparations from five to six animals (*P<0.01, two-tailed Student's *t* test)

ditions, the release of glutamate evoked by 4-AP (1 mM) was 1.5±0.2 nmol/mg/5 min. This Ca^{2+}-independent release evoked by 4-AP was reduced by DL-TBOA (10 μM), a non-selective inhibitor of all excitatory amino acid transporter (EAAT) subtypes (0.3±0.1 nmol/mg/5 min; n=5; P<0.05). However, idebenone (50 μM) did not affect the Ca^{2+}-independent component of 4-AP-evoked glutamate release (1.3±0.2 nmol/mg/5 min) (n=5; Fig. 2A). The next series of experiments was carried out with the normal Ca^{2+} concentration. In Fig. 2B and D, the 4-AP (1 mM)-evoked glutamate release was inhibited by idebenone (50 μM) to about the same extent both in the absence and presence of DL-TBOA (10 μM). DL-TBOA by itself almost doubled the 4-AP (1 mM)-evoked glutamate release. Finally, we investigated the action of idebenone in the presence of bafilomycin A1, which causes the depletion of glutamate in synaptic vesicles. Figure 2C shows that bafilomycin A1 (0.1 μM) reduced control 1 mM 4-AP-evoked glutamate release to 2.7±0.1 nmol/mg/5 min (P<0.01), and completely prevented the

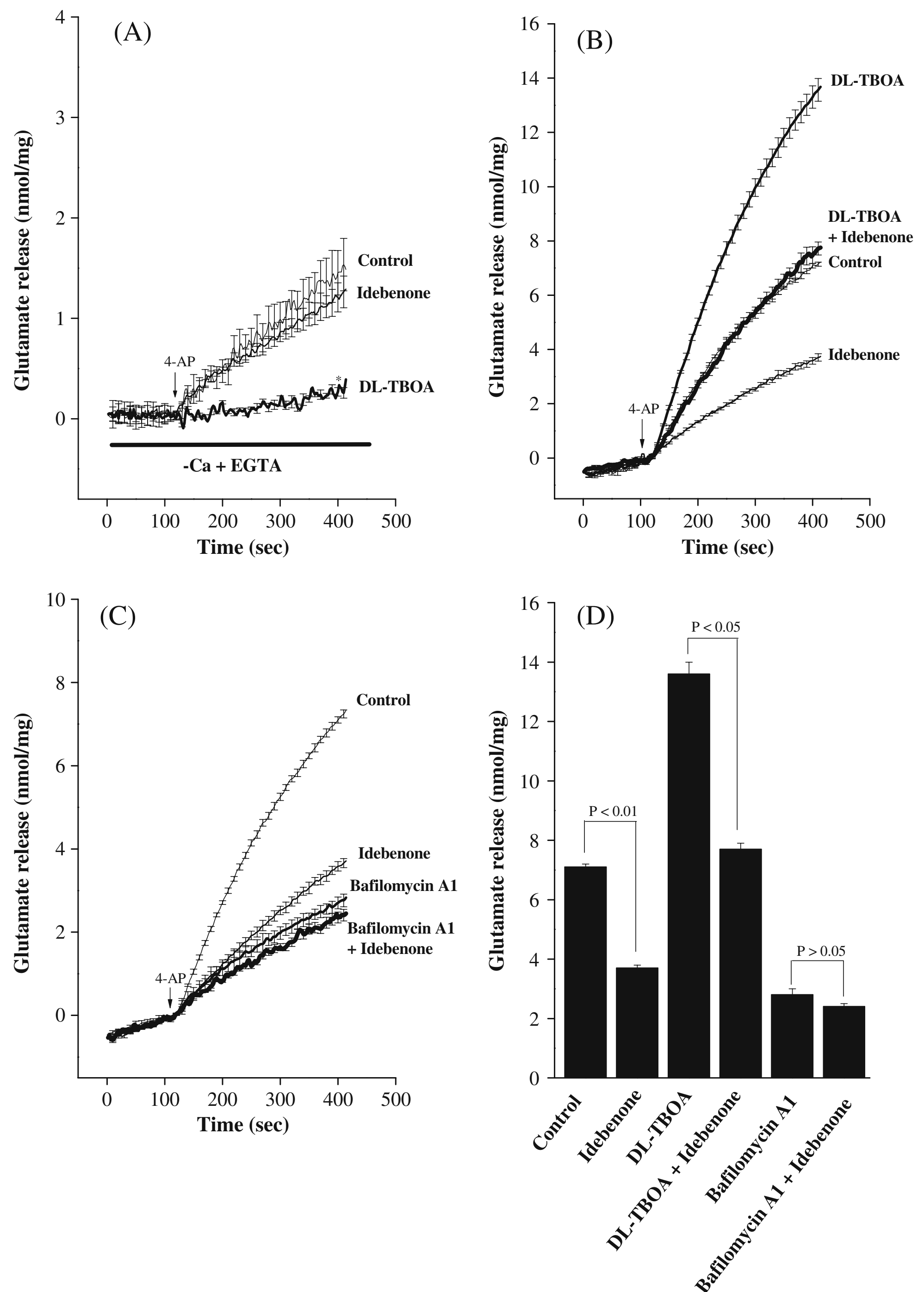

Fig. 2 Idebenone-mediated inhibition of 4-AP-induced glutamate release is due to a decrease in vesicular exocytosis. **A** Ca^{2+}-independent release was assayed by omitting $CaCl_2$ and adding 300 μM EGTA 10 min prior to depolarization, and was evoked by 1 mM 4-APunder control conditions, or in the presence of 10 μM DL-TBOA or 50 μM idebenone added 10 min prior to the addition of 4-AP. **B** and **C** Glutamate release was evoked by 1 mM 4-AP in the absence (control) or presence of 50 μM idebenone, 10 μM DL-TBOA, 10 μM DL-TBOA+50 μM idebenone, 0.1 μM bafilomycin A1, or 0.1 μM bafilomycin A1+50 μM idebenone. Idebenone was added 10 min before depolarization and, DL-TBOA or bafilomycin A1, 10 min prior to this. **D** Quantitative comparison of the extent of glutamate release by 1 mM 4-AP in the absence and presence of 50 μM idebenone, and absence and presence of 10 μM DL-TBOA, or 0.1 μM bafilomycin A1. Results are the mean±SEM values of independent experiments, using synaptosomal preparations from five to six animals

inhibitory effect of idebenone (50 μM) on 4-AP-evoked glutamate release (2.4±0.4 nmol/mg/5 min; n=6; Fig. 2C). In the presence of bafilomycin A1, therefore, idebenone failed to inhibit the 4-AP-induced glutamate release (P> 0.05; one-way repeated-measures ANOVA; Fig. 2D). All these results suggest that the idebenone-mediated inhibition of 4-AP-evoked glutamate release is mediated by a reduction in the Ca^{2+}-dependent exocytotic component of glutamate release.

Effect of idebenone on cytosolic Ca^{2+} levels and synaptosomal membrane potential

To further test whether a reduction in $[Ca^{2+}]_C$ is responsible for the idebenone-mediated inhibition of release, we used a Ca^{2+} indicator Fura-2 to monitor intraterminal Ca^{2+} levels directly. Under control conditions, 4-AP (1 mM) caused a rise in $[Ca^{2+}]_C$, from 135.3±1.9 nM to a plateau level of 200.1±4.5 nM. Application of idebenone (50 μM) did not significantly affect basal Ca^{2+} levels (134.7±2.1 nM), but caused a ~51% decrease in the 4-AP-evoked rise in $[Ca^{2+}]_C$(166.7±3.8 nM; P<0.01; n=5; Fig. 3). Because inhibition of Na^+ channels or activation of K^+ channels is known to stabilize membrane excitability and consequently cause a reduction in the intracellular Ca^{2+} and neurotransmitter release (Pongs et al. 1999; Rehm and Tempel 1991; Li et al. 1993), we reasoned that the observed inhibitory effect of idebenone on 4-AP-induced increase in $[Ca^{2+}]_C$ could be due to an alteration of nerve terminal excitability. To test this possibility, we examined the effect of idebenone on the synaptosomal plasma membrane potential under resting conditions and on depolarization with the membrane-potential-sensitive dye $DiSC_3(5)$. As shown in Fig. 4A, 4-AP (1 mM) caused an increase in $DiSC_3(5)$ fluorescence of 12.2±0.5 fluorescence units/5 min. Preincubation of synaptosomes with idebenone(50 μM) for 10 min before 4-AP addition did not alter the resting membrane potential, and produced no significant change in the 4-AP-mediated increase in $DiSC_3(5)$ fluorescence (12.4±0.4 fluorescence units/5 min; P>0.05; n=5). This indicates that the observed inhibition of evoked glutamate release by idebenone is unlikely to have been caused by a hyperpolarizing effect of the drug on the synaptosomal plasma membrane potential, or attenuation of the depolarization produced by 4-AP. To confirm this, we examined the effect of idebenone on the release of glutamate evoked by an alternative secretagogue, high external $[K^+]$ (Fig. 4B). Elevated extracellular KCl depolarizes the plasma membrane by shifting the K^+ equilibrium potential above the threshold potential for activation of voltage-dependent ion channels. Whereas Na^+ channels are inactivated under these conditions, VDCCs are activated nonetheless to mediate Ca^{2+} entry, which supports neurotransmitter release (Barrie et al. 1991). In Fig. 4B, addition of 15 mM KCl led to a glutamate release of 9.5± 0.1 nmol/mg/5 min, which was reduced to 6.1±0.2 nmol/mg/5 min in the presence of 50 μM idebenone (P<0.01; n=5).

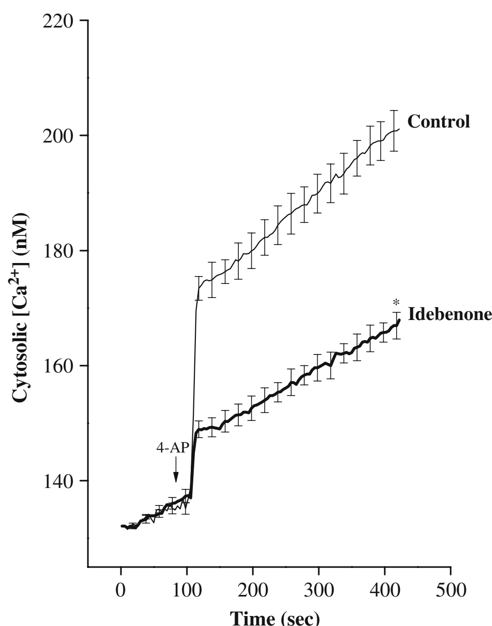

Fig. 3 Idebenone reduces the 4-AP-induced increase in cytosolic Ca^{2+} ($[Ca^{2+}]_C$). $[Ca^{2+}]_C$ was monitored using Fura-2. The synaptosomes were stimulated using 1 mM 4-AP in the absence (control) or presence of 50 μM idebenone, which was added 10 min before stimulation. Results are the mean±SEM values of independent experiments, using synaptosomal preparations from five animals. The 4-AP-induced increase in $[Ca^{2+}]_C$ levels in the presence of idebenone was significantly different from that induced in the absence of idebenone (*P<0.01, two-tailed Student's t test)

Effect of ω-CgTX MVIIC, dantrolene, and CGP37157 on inhibition of 4-AP-evoked glutamate release by idebenone

In the adult rat cerebrocortical nerve terminal preparation, the release of glutamate evoked by depolarization is reported to cause Ca^{2+} influx through $Ca_v2.2$ (N-type) and $Ca_v2.1$ (P/Q-type) channels and Ca^{2+} release from internal stores (Berridge 1998; Millan and Sanchez-Prieto 2002; Schenning et al. 2006). Therefore, we next sought to establish which part of Ca^{2+} source was involved in the idebenone-mediated inhibition of 4-AP-evoked glutamate release. To assess the role of $Ca_v2.2$ and $Ca_v2.1$ channels, synaptosomes were preincubated with 2 μM ω-conotoxin MVIIC (ω-CgTX MVIIC), a wide-spectrum blocker of $Ca_v2.2$ and $Ca_v2.1$ channels. In Fig. 5A, glutamate release of 7.3±0.1 nmol/mg/5 min under control conditions was significantly decreased to 3.6±0.2 nmol/mg/5 min by idebenone (50 μM). Glutamate release was also significantly reduced by ω-CgTX MVIIC (2 μM) to 2.5±

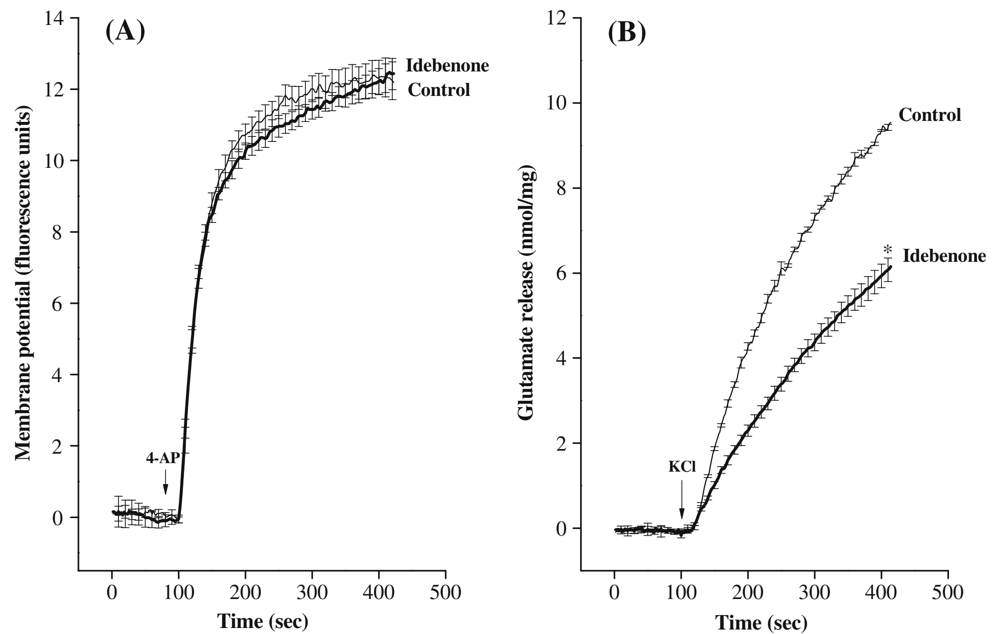

Fig. 4 Idebenone does not alter the synaptosomal membrane potential. **A** Synaptosomal membrane potential was monitored with 5 μM $DiSC_3(5)$ on depolarization with 1 mM 4-AP, in the absence (control) or presence of 50 μM idebenone added 10 min before depolarization. **B** Glutamate release was evoked by 15 mMKCl in the absence or presence of 50 μM idebenone, added 10 min before depolarization. Results are the mean± SEM values of independent experiments, using synaptosomal preparations from five animals

0.3 nmol/mg/5 min ($P<0.01$). In the presence of ω-CgTX MVIIC, 50 μM idebenone failed to decrease the 4-AP-induced glutamate release ($n=9$). In addition, a potential role of intracellular Ca^{2+} release in idebenone-mediated inhibition of glutamate release was tested in the presence of dantrolene, an inhibitor of intracellular Ca^{2+} release from

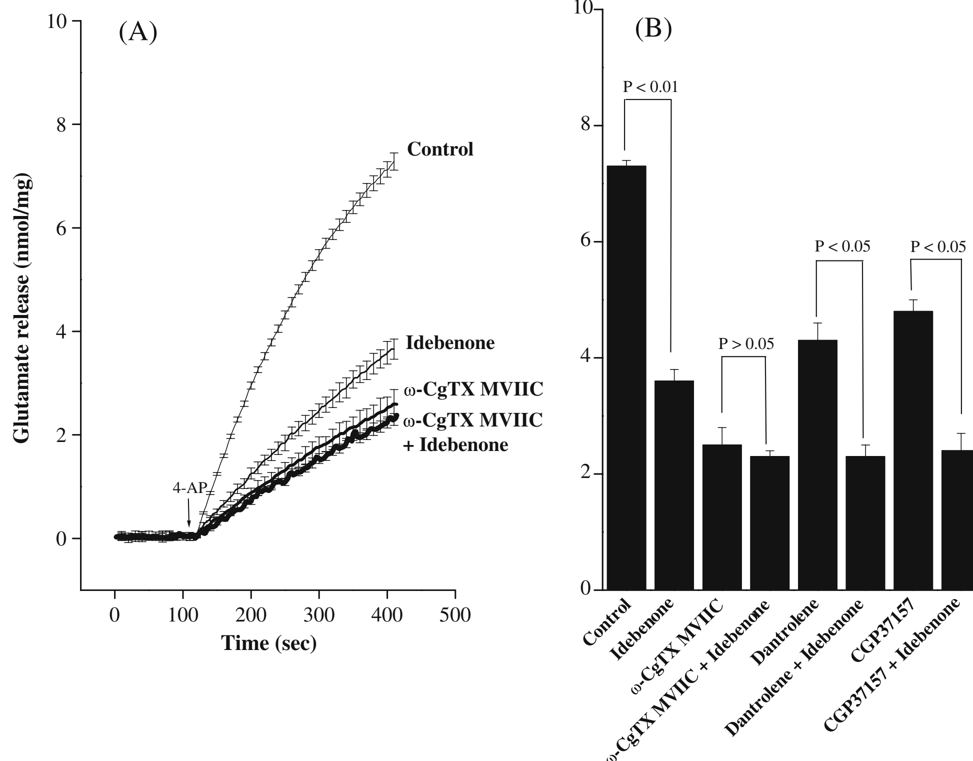

Fig. 5 Suppression of idebenone-mediated inhibition of glutamate release by the $Ca_v2.2$ and $Ca_v2.1$ channel blocker ω-CgTX MVIIC. **A** Glutamate release was evoked by 1 mM 4-AP in the absence (control) or presence of 50 μM idebenone, 2 μM ω-CgTX MVIIC, or 2 μM ω-CgTXMVIIC+50 μM idebenone. **B** Quantitative comparison of the extent of glutamate release by 1 mM 4-AP in the absence and presence of 50 μM idebenone, and absence and presence of 2 μM ω-CgTX MVIIC, 100 μM dantrolene, or 100 μM CGP37157. Idebenone was added 10 min before depolarization and other drugs 30 min prior to this. Results are the mean±SEM values of independent experiments, using synaptosomal preparations from six to nine animals

endoplasmic reticulum, and 7-chloro-5-(2-chloropheny)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP37157), a membrane-permeant blocker of mitochondrial Na^+/Ca^{2+} exchange. Dantrolene (100 μM) reduced control 4-AP-evoked release from 7.2±0.1 to 4.3±0.3 nmol/mg/5 min indicating that intracellular Ca^{2+} release directly supports glutamate release from synaptosomes. In the presence of dantrolene, however, idebenone (50 μM) still effectively inhibited 4-AP-evoked glutamate release to 2.3±0.2 nmol/mg/5 min (n=6; P<0.05; Fig. 5B). In six synaptosomal preparations tested, idebenone induced a 46.5±5.3% decrease in the 4-AP-evoked glutamate release after application of dantrolene, which was not significantly different from the inhibition produced by idebenone alone (50.7±3.4%; P>0.05). Similarly with dantrolene, CGP37157 (100 μM) decreased the release of glutamate evoked by 4-AP (1 mM) without affecting the extent of the idebenone-mediated inhibition of 4-AP-evoked glutamate release (n=6; Fig. 5B).

Effect of H89 or GF109203X on inhibition of 4-AP-evoked glutamate release by idebenone

Since the PKA or the PKC signaling cascade is known to be present at the presynaptic level and has a crucial role in neurotransmitter exocytosis (Vaughan et al. 1998; Millan et al. 2003; Wang and Sihra 2003), we investigated whether the cascade(s) participated in idebenone-mediated inhibition of glutamate release. To this end, we studied the effect of idebenone following inhibition of PKA or PKC. In Fig. 6A and B, the two PKA inhibitors H89 (100 μM) and KT5720 (100 μM) as well as the two PKC inhibitors GF109203X (10 μM) and Ro318220 (10 μM) by themselves reduced the 4-AP-evoked glutamate release by about 50%, suggesting basal PKA and PKC activity, respectively. However, the idebenone-mediated inhibition of 4-AP-evoked glutamate release was affected in a qualitatively different manner by the protein kinase inhibitors. The idebenone-mediated inhibition of 4-AP-evoked glutamate release was abolished by either PKA inhibitor (Fig. 6A and B). On the other hand, the inhibitory effect of idebenone on the 4-AP-evoked glutamate release was not affected by either PKC inhibitor (Fig. 6B). We also examined the effect of idebenone on 4-AP-evoked rise in $[Ca^{2+}]_C$ in the presence of PKA inhibitors, H89 and KT5720. The latter two drugs by themselves decreased the 4-AP (1 mM)-evoked rise in $[Ca^{2+}]_C$ from 206.3±3.5 nM to 181.1±5.1 nM and 177.3±3.8 nM, respectively (P<0.01; n=5). In the presence of H89 (100 μM), the idebenone-induced inhibition of 4-AP-evoked rise in $[Ca^{2+}]_C$ was completely prevented (n=5; Fig. 6C). A similar result was observed with KT5720 (100 μM; n=5; Fig. 6C). These results suggest that idebenone-inhibited Ca^{2+} influx and glutamate release involves a PKA pathway.

Discussion

It is known that excessive release of glutamate is a critical factor in the neuropathology of acute and chronic brain disorders (Obrenovitch and Urenjak 1997; Meldrum 2000; Raiteri et al. 2004). Although idebenone has been shown to protect neurons against glutamate-induced excitotoxicity in several in vitro and in vivo experimental studies (Miyamoto et al. 1989; Miyamoto and Coyle 1990; Bruno et al. 1994), there are no previous studies about the effect of idebenone on glutamate release in central neurons. Thus, the purpose of this study was to investigate the relationship between idebenone and presynaptic modulation of glutamate release and to determine the underlying molecular mechanisms. By using a preparation of nerve terminals from rat cerebral cortex and examining the release of endogenous glutamate, we demonstrated that idebenone rapidly reduced depolarization-evoked glutamate release in a dose-dependent manner. To our knowledge, this is the first report of a significant effect of idebenone on the central glutamate system. Several possible mechanisms for this effect are discussed below.

The release of glutamate as a result of the depolarization of isolated nerve terminals has two components. The first is a physiologically relevant Ca^{2+}-dependent component, which is produced by exocytosis of synaptic vesicles that contain glutamate. The second is a Ca^{2+}-independent component that results from prolonged depolarization that causes a membrane-potential-mediated shift of the glutamate transporter steady-state toward the outward direction, to affect cytosolic glutamate efflux (Nicholls et al. 1987). In the present study, the Ca^{2+}-dependent component of glutamate release is the molecular basis for the idebenone-mediated inhibition of 4-AP-evoked glutamate release. This conclusion is based on the following observations. Firstly, idebenone did not affect 4-AP-evoked glutamate release in the absence of extracellular Ca^{2+} (Ca^{2+}-independent release). Secondly, the inhibitory effect of idebenone on 4-AP-evoked glutamate release was effectively abolished by bafilomycin A1, which depletes the glutamate content of synaptic vesicles, but not by DL-TBOA, a non-selective inhibitor of all EAAT subtypes.

In principle, neurotransmitter release can be modulated at several loci in the stimulus-exocytosis cascade, including ion-channels modulating nerve terminal excitability, VDCCs, downstream of Ca^{2+} entry, components of the synaptic vesicle trafficking and exocytotic apparatus (Sihra and Nichols 1993; Thompson et al. 1993; Nicholls 1998). Thus, inhibition of Ca^{2+}-dependent glutamate release by idebenone could be ascribed to an alteration of plasma membrane potential, and/or a direct inhibition of the exocytosis-coupled Ca^{2+} channel. Our demonstration, using the Ca^{2+} indicator Fura-2, that the 4-AP-evoked increase in $[Ca^{2+}]_C$ was reduced by idebenone suggests that idebenone

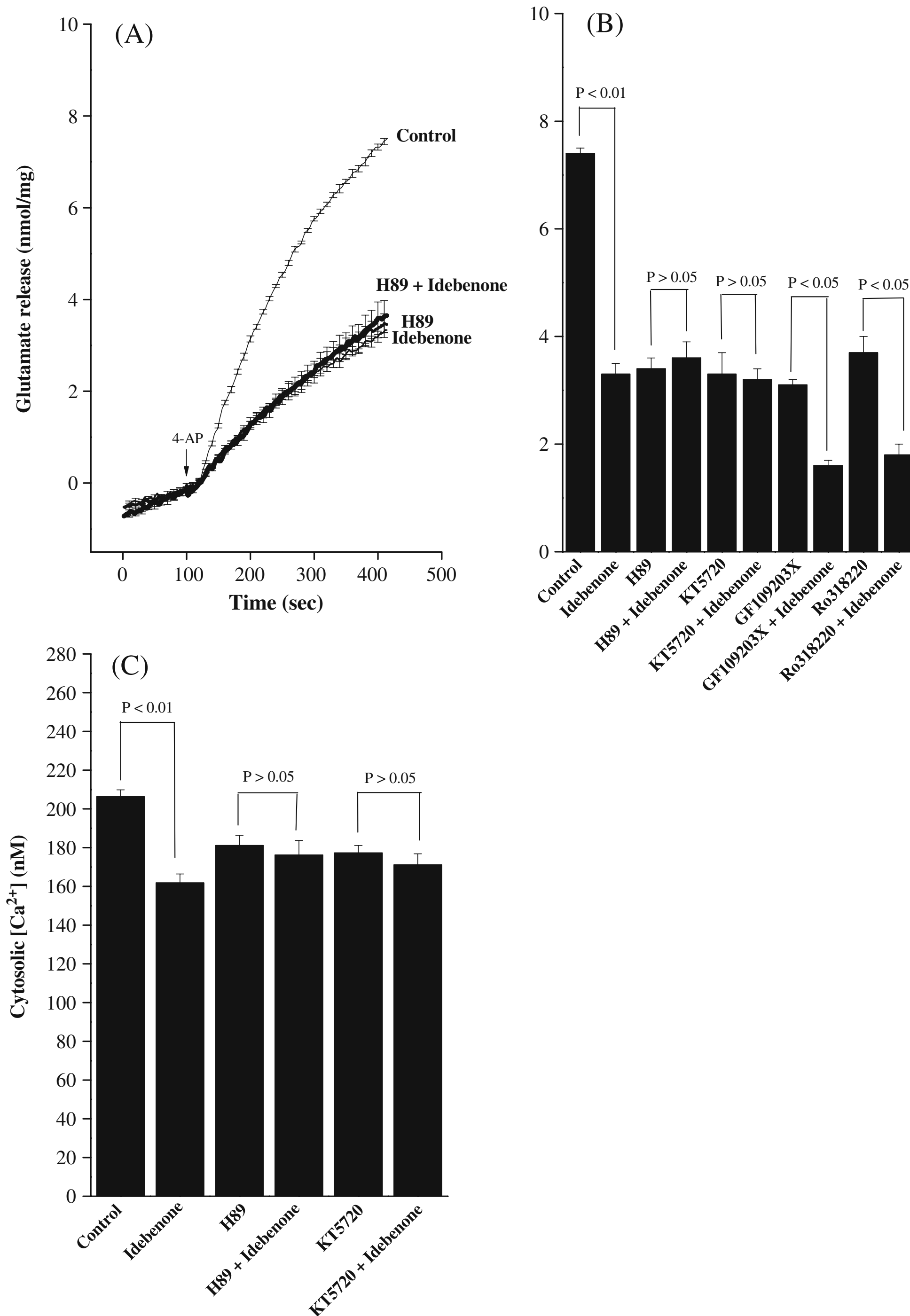

Fig. 6 Idebenone-mediated inhibition of 4-AP-induced Ca^{2+} influx and glutamate release is prevented by thePKA inhibitors, H89 and KT5720. **A** Glutamate release was evoked by 1 mM 4-AP in the absence (control) or presence of 50 μM idebenone, 100 μM H89, or 100 μM H89+50 μM idebenone. **B** Quantitative comparison of the extent of glutamate release by 1 mM 4-AP in the absence and presence of 50 μM idebenone, and absence and presence of 100 μM H89, 100 μM KT5720, 10 μM GF109203X, or 10 μM Ro318220. **C** The elevation of cytosolic Ca^{2+} levels was observed 5 min after depolarization induced by 1 mM 4-AP, in the absence (control) or presence of 50 μM idebenone, 100 μM H89, 100 μM H89 and 50 μM idebenone, 100 μM KT5720, or 100 μM KT5720 and 50 μM idebenone. Idebenone was added 10 min before depolarization and H89, KT5720, GF109203X, or Ro31822030 min prior to this. Results are the mean±SEM values of independent experiments, using synaptosomal preparations from five to six animals

attenuates glutamate release by indirectly or directly decreasing intracellular Ca^{2+} levels. In synaptic terminals, a depolarization-induced increase in $[Ca^{2+}]_C$, coupled with glutamate release, is mediated by Ca^{2+} influx through $Ca_v2.2$ and $Ca_v2.1$ channels and Ca^{2+} release from intracellular stores, such as the endoplasmic reticulum and mitochondria (Berridge 1998; Millan and Sanchez-Prieto 2002). In this study, the inhibition of glutamate release by idebenone was highly sensitive to the inhibition of $Ca_v2.2$ and $Ca_v2.1$ channels, which are involved in triggering glutamate release from synaptosomes (Vazquez and Sanchez-Prieto 1997; Millan and Sanchez-Prieto 2002). This suggested that $Ca_v2.2$ and $Ca_v2.1$ channels are involved in the modulation of glutamate release by idebenone. However, since the suppression of $Ca_v2.2$ and $Ca_v2.1$ channel activity did not completely abolish the inhibitory effect of idebenone on 4-AP-evoked glutamate release (about 8% of the activity remained), we cannot rule out the involvement of other types of Ca^{2+} channels. In contrast, the participation of reduced release of stored Ca^{2+} from the endoplasmic reticulum and mitochondria during the idebenone-mediated inhibition of glutamate release could be excluded. This was because the inhibitory effect of idebenone on 4-AP-evoked glutamate release was not affected by dantrolene, an inhibitor of intracellular Ca^{2+} release from the endoplasmic reticulum, and by CGP37157, a membrane-permeant blocker of mitochondrial Na^+/Ca^{2+} exchange. In this regard, in the presence of dantrolene or CGP37157, idebenone still induced a 47% or 49% decrease in the glutamate release, respectively. The additive relationship between dantrolene or CGP37157 and idebenone indicates that although intracellular Ca^{2+} release contributes significantly to the 4-AP-evoked glutamate release, they appear not to mediate the effect of idebenone on glutamate release. These results therefore imply that the inhibition of glutamate release by idebenone occurs primarily via a suppression of Ca^{2+} influx through $Ca_v2.2$ and $Ca_v2.1$ channels. Although we do not have direct evidence that idebenone acts on presynaptic Ca^{2+} channels, our finding is consistent with a previous study, which demonstrated that idebenone inhibits catecholamine secretion by suppressing Ca^{2+} influx through VDCCs in cultured adrenal chromaffin cells (Houchi et al. 1991).

The question remained as to whether the suppression of release-coupled VDCCs by idebenone reflected a direct effect on VDCC function or secondary effects resulting from an alteration in plasma membrane potential. In this regard, the latter possibility is unlikely for three reasons. First, no significant effect of idebenone on synaptosomal plasma membrane potential, measured with a membrane-potential-sensitive dye, $DiSC_3(5)$, was observed either in the resting condition or on depolarization with 4-AP (indicating a lack of effect on K^+ conductance). Second, idebenone significantly inhibited both 4-AP and KCl-evoked glutamate release. Since 4-AP-evoked glutamate release involves Na^+ and Ca^{2+} channels whereas KCl-evoked glutamate release only involves Ca^{2+} channels (Barrie et al. 1991; Nicholls 1998), it is not likely that Na^+ channels are involved in the modulation of glutamate release by idebenone. Third, idebenone did not affect the 4-AP-evoked Ca^{2+}-independent glutamate release, which is only dependent on the membrane potential (Attwell et al. 1993). Therefore, our observations clearly suggest that idebenone is able to inhibit glutamate release specifically and directly via the reducing activity of the VDCCs, rather than any upstream effect on nerve terminal excitability as a result of modulation of Na^+ or K^+ ion channels.

Many studies have shown that protein kinases, such as PKC and PKA, are involved in the regulation of presynaptic VDCCs function and glutamate release (Herrero and Sanchez-Prieto 1996; Vaughan et al. 1998; Wang et al. 2002; Millan et al. 2003; Wang and Sihra 2003). As a result, it is likely that a protein kinase signaling pathway is involved in the modulation of glutamate release by idebenone. In elucidating this, we found that the inhibitory effects of idebenone on 4-AP evoked rise in $[Ca^{2+}]_C$ and glutamate release were prevented by the PKA inhibitors H89 and KT5720. This suggests that an intracellular cascade involving the suppression of PKA-dependent pathways is linked to inhibition of 4-AP-evoked glutamate release by idebenone. Moreover, the inhibitory effect of idebenone on 4-AP-evoked glutamate release was not affected by the PKC inhibitors GF109203X and Ro318220, which demonstrated some specificity for idebenone action on the PKA pathway. In fact, in addition to the phosphorylation of presynaptic VDCCs, a number of synaptic proteins associated with moving, docking, and fusion of synaptic vesicle, including synapsin I, rabphilin3A, synaptobrevin, syntaxin, and SNAP-25, have been reported to be phosphorylated by PKA. Such phosphorylation reaction enhances vesicle mobilization to the plasma membrane and neurotransmitter release (Greengard et al. 1993; Fykse et al. 1995; Lonart et al. 1998; Risinger and Bennett 1999). Whether idebenone inhibits glutamate release by decreasing PKA-dependent phosphorylation of synaptic proteins and the availability of synaptic vesicles should be considered.

In the current study, idebenone inhibited glutamate release in cerebral cortex synaptosomes at the relatively high concentration of 50 μM. Several studies have reported that idebenone, at 50–100 μM, increases adenosine levels in hippocampal slices after ischemia-like conditions and reduces free radical formation in rat brain synaptosomes (Latini et al. 1993; Cardoso et al. 1998). The present study is consistent with these reports, however, the antioxidant and neural protective effects of idebenone were observed in

a lower concentration range, i.e., 0.1–10 μM (Miyamoto et al. 1989; Murphy et al. 1990; Oka et al. 1993; Hirai et al. 1998; Pereira et al. 1999).

In conclusion, the main finding of the present study is that, in rat cerebrocortical nerve terminals, idebenone decreases glutamate release through the suppression of presynaptic $Ca_v2.2$ and $Ca_v2.1$ channels and PKA activity. This phenomenon might account for some of its neuroprotective effect in several neuronal injuries in vitro, especially in glutamate-induced neurotoxicity; this is because excessive glutamate release has been proposed to be involved in the pathophysiology of several neurological states, including ischemic brain damage and neurodegenerative diseases (Obrenovitch and Urenjak 1997; Meldrum 2000). Although the mechanisms for the neuroprotection conferred by idebenone are still under investigation, the present finding may provide further understanding of the mode of idebenone action in the brain and highlights the therapeutic potential of this drug in the treatment of a wide range of neurological and neurodegenerative disorders.

Acknowledgements This study was supported by a grant from the Shin Kong Wu Ho-Su Memorial Hospital (99SKH-FJU-11). We thank Sin-Yi Tsai for expert technical assistance.

References

Akerman KE, Scott IG, Heikkila JE, Heinonen E (1987) Ionic dependence of membrane potential and glutamate receptor-linked responses in synaptoneurosomes as measured with a cyanine dye, $DiSC_2(5)$. J Neurochem 48:552–559

Attwell D, Barbour B, Szatkowski M (1993) Nonvesicular release of neurotransmitter. Neuron 375:645–653

Barrie AP, Nicholls DG, Sanchez-Prieto J, Sihra TS (1991) An ion channel locus for the protein kinase C potentiation of transmitter glutamate release from guinea pig cerebrocorticalsynaptosomes. J Neurochem 57:1398–1404

Berridge MJ (1998) Neuronal calcium signaling. Neuron 21:13–26

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Bruno V, Battaglia G, Copani A, Sortino MA, Canonico PL, Nicoletti F (1994) Protective action of idebenone against excitotoxic degeneration in cultured cortical neurons. Neurosci Lett 178:193–196

Cardoso SM, Pereira C, Oliveira CR (1998) The protective effect of vitamin E, idebenone and reduced glutathione on free radical mediated injury in rat brain synaptosomes. Biochem Biophys Res Commun 246:703–710

Coyle JT, Puttfarcken P (1993) Oxidative stress, glutamate, and neurodegenerative disorders. Science 262:689–695

Danbolt NC (2001) Glutamate uptake. ProgNeurobiol 65:1–105

Dunkley PR, Jarvie PE, Heath JW, Kidd GJ, Rostas JA (1986) A rapid method for isolation of synaptosomes on Percoll gradients. Brain Res 372:115–129

Fykse EM, Li C, Sudhof TC (1995) Phosphorylation of rabphilin-3A by Ca2+/calmodulin- and cAMP-dependent protein kinases in vitro. J Neurosci 15:2385–2395

Greenamyre JT, Porter RH (1994) Anatomy and physiology of glutamate in the CNS. Neurology 44:S7–S13

Greengard P, Valtorta F, Czernik AJ, Benfenati F (1993) Synaptic vesicle phosphoproteins and regulation of synaptic function. Science 259:780–785

Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450

Herrero I, Sanchez-Prieto J (1996) cAMP-dependent facilitation of glutamate release by β-adrenergic receptor in cerebrocortical nerve terminals. J Neurochem 271:30554–30560

Hirai K, Hayako H, Kato K, Miyamoto M (1998) Idebenone protects hippocampal neurons against amyloid β-peptide-induced neurotoxicity in rat primary cultures. Naunyn-Schmiedeberg's Arch Pharmacol 358:582–585

Houchi H, Azuma M, Oka M, Morita K (1991) Idebenone inhibits catecholamine secretion through its blocking action on Ca^{2+} channels in cultured adrenalchromaffin cells. Jpn J Pharmacol 57:553–558

Latini S, Pedata F, Pepeu G (1993) Effect of idebenone on adenosine outflow and adenine uncleotidw level in hippocampal slices under ischemia-like conditions. Eur J Pharmacol 249:65–70

Li M, West JW, Numann R, Murphy BJ, Scheuer T, Catterall WA (1993) Convergent regulation of sodium channels by protein kinase C and cAMP-dependent protein kinase. Science 261:1439–1442

Lonart G, Janz R, Johnson KM, Sudhof TC (1998) Mechanism of action of rab3A in mossy fiber LTP. Neuron 21:1141–1150

McMahon HT, Nicholls DG (1991) Transmitter glutamate release from isolated nerve terminals: evidence for biphasic release and triggering by localized Ca^{2+}. J Neurochem 56:86–94

Meldrum BS (2000) Glutamate as a neurotransmitter in the brain: review of physiology and pathology. J Nutr 130:1007S–1015S

Millan C, Sanchez-Prieto J (2002) Differential coupling of N and P/Q-type calcium channels to glutamate exocytosis in the rat cerebral cortex. Neurosci Lett 330:29–32

Millan C, Torres M, Sanchez-Prieto J (2003) Co-activation of PKA and PKC in cerebrocortical nerve terminals synergistically facilitates glutamate release. J Neurochem 87:1101–1111

Miyamoto M, Coyle JT (1990) Idebenone attenuates neuronal degeneration induced by intrastriatal injection of excitotoxins. Exp Neurol 108:38–45

Miyamoto M, Murphy TH, Schnaar RL, Coyle JT (1989) Antioxidants protect against glutamate-induced cytotoxicity in a neuronal cell line. J Pharmacol Exp Ther 250:1132–1140

Murphy TH, Schnaar RL, Coyle JT (1990) Immature cortical neurons are uniquely sensitive to glutamate toxicity by inhibiton of cysteine uptake. FASEB J 4:1624–1633

Nagai Y, Yoshida K, Narumi S, Tanayama S, Nagaoka A (1989) Brain distribution of idebenone and its effect on local blood glucose utilization in rats. Arch Gerontol Geriatr 8:257–272

Nagaoka A, Suno M, Shibota M, Kakihana M (1989) Effects of idebenone on neurological deficits, local cerebral blood flow, and energy metabolism in rats with experimental cerebral ischemia. Arch Gerontol Geriatr 8:193–202

Nappi G, Bono G, Merlo P, Denaro A, Proietti R, Martucci N, Fioravanti M (1992) Long-term idebenone treatment of vascular and degenerative brain disorders of the elderly. Arch Gerontol Geriatr 15:261–269

Nicholls DG (1998) Presynaptic modulation of glutamate release. Prog Brain Res 116:15–22

Nicholls DG, Sihra TS (1986) Synaptosomes possess an exocytotic pool of glutamate. Nature 321:772–773

Nicholls DG, Sihra TS, Sanchez-Prieto J (1987) Calcium-dependent and -independent release of glutamate from synaptosomes monitored by continuous fluorometry. J Neurochem 49:50–57

Obrenovitch TP, Urenjak J (1997) Altered glutamatergic transmission in neurological disorders: from high extracellular glutamate to excessive synaptic efficacy. Prog Neurobiol 51:39–87

Oka A, Belliveau MJ, Rosenberg PA, Volpe JJ (1993) Vulnerability of oligodendroglia to glutamate: pharmacology, mechanisms, and prevention. J Neurosci 13:1441–1453

Pelleymounter MA, Cullen MJ (1993) Effects of idebenone on information processing in ages Long-Evans rats. Pharmacol Biochem Behav 46:415–421

Pereira C, Santos MS, Oliveira C (1999) Involvement of oxidative stress on the impairment of energy metabolism induced by A beta peptides on PC12 cells: protection by antioxidants. Neurobiol Dis 6:209–219

Pongs O, Leicher T, Berger M, Roeper J, Bahring R, Wray D et al (1999) Functional and molecular aspects of voltage-gated K^+ channel beta subunits. Ann N Y Acad Sci 868:344–355

Raiteri L, Stigliani S, Zappettini S, MercuriNB RM, Bonanno G (2004) Excessive and precocious glutamate release in a mouse model of amyotrophic lateral sclerosis. Neuropharmacology 46:782–792

Rawls SM, McGinty JF, Terrian DM (1999) Presynaptic κ-opioid muscarinic receptors inhibit the calcium-dependent component of evoked glutamate release from striatal synaptosomes. J Neurochem 73:1058–1065

Rehm H, Tempel BL (1991) Voltage-gated K^+ channels of mammalian brain. FASEB J 5:164–170

Risinger C, Bennett MK (1999) Differential phosphorylation of syntaxin and synaptosome-associated protein of 25 kDa (ANAP-25) isoforms. J Neurochem 72:614–624

Schenning M, Proctor DT, Ragnarsson L, Barbier J, Lavidis NA, Molgo JJ et al (2006) Glycerotoxin stimulates neurotransmitter release from N-type Ca^{2+} channel expressing neurons. J Neurochem 98:894–904

Schinder AF, Olson EC, Spitzer NC, Montal M (1996) Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. J Neurosci 16:6125–6133

Senin U, Parnetti L, Barbagallo-Sangiorgi G, Bartorelli L, Bocola V, Capurso A, Cuzzupoli M, Denaro M, Marigliano V, Tammaro AE, Fioravanti M (1992) Idebenone in senile dementia of Alzheimer type: a multicentre study. Arch Gerontol Geriatr 15:249–260

Sihra TS, Nichols RA (1993) Mechanisms in the regulation of neurotransmitter release from brain nerve terminals: current hypotheses. Neurochem Res 18:47–58

Sihra TS, Bogonez E, Nicholls DG (1992) Localized Ca^{2+} entry preferentially effects protein dephosphorylation, phosphorylation, and glutamate release. J Biol Chem 267:1983–1989

Suno M, Nagaoka A (1984) Inhibition of lipid peroxidation by a novel compound (CV-2619) in brain mitochondria and mode of action of the inhibition. Biochem Biophys Res Commun 125:1046–1052

Thompson SM, Capogna M, Scanziani M (1993) Presynaptic inhibition in the hippocampus. Trends Neurosci 16:222–227

Vaughan PF, Walker JH, Peers C (1998) The regulation of neurotransmitter secretion by protein kinase C. Mol Neurobiol 18:125–155

Vazquez E, Sanchez-Prieto J (1997) Presynaptic modulation of glutamate release targets different calcium channels in rat cerebrocortical nerve terminals. Eur J Neurosci 9:2009–2018

Wang SJ, Sihra TS (2003) Opposing facilitatory and inhibitory modulation of glutamate release elicited by cAMP production in cerebrocortical nerve terminals (synaptosomes). Neuropharmacology 44:686–697

Wang SJ, Sihra TS (2004) Noncompetitive metabotropic glutamate5 receptor antagonist (E)-2-methyl-6-styryl-pyridine (SIB1893) depresses glutamate release through inhibition of voltage-dependent Ca^{2+} entry in rat cerebrocortical nerve terminals (synaptosomes). J Pharmacol Exp Ther 309:951–958

Wang SJ, Coutinho V, Sihra TS (2002) Presynaptic cross-talk of β-adrenoreceptor and 5-hydroxytryptamine receptor signaling in the modulation of glutamate release from cerebrocortical nerve terminals. Br J Pharmacol 137:1371–1379

Weyer G, Babej-Dölle RM, Hadler D, Hofmann S, Herrmann WM (1997) A controlled study of 2 doses of idebenone in the treatment of Alzheimer's disease. Neuropsychobiology 36:73–82

Yamada K, Tanaka T, Han D, Senzaki K, Kameyama T, Nabeshima T (1999) Protective effects of idebenone and α-tocopherol on β-amyloid-(1–42)-induced learning and memory deficits in rats: implication of oxidative stress in β-amyloid-induced neurotoxicity in vivo. Eur J Neurosci 11:83–90

Yamazaki N, Kiyota Y, Take Y, Miyamoto M, Nagawa Y, Nagaoka A (1989) Effects of idebenone on memory impairment induced in ischemic and embolization models of cerebrovascular disturbance in rats. Arch Gerontol Geriatr 8:213–224

Yang TT, Wang SJ (2009) Pyridoxine inhibits depolarization-evoked glutamate release in nerve terminals from rat cerebral cortex: a possible neuroprotective mechanism? J Pharmacol Exp Ther 331:244–253

Zs.-Nagy I (1990) Chemistry, toxicology, pharmacology and pharmacokinetics of idebenone: a review. Arch Gerontol Geriatr 11:177–186