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Quercetin inhibits depolarization-evoked glutamate release in nerve terminals from rat cerebral cortex

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ABSTRACT

Quercetin, a naturally occurring flavonoid, has been reported to have a neuroprotective profile. An excessive release of glutamate is widely considered to be one of the molecular mechanisms of neuronal damage in several neurological diseases. This study investigated whether quercetin affected glutamate release in rat cerebral cortex nerve terminals (synaptosomes) and explored the possible mechanism. Quercetin inhibited the release of glutamate evoked by the K^+ channel blocker 4-aminopyridine (4-AP), and this effect was prevented by the chelating extracellular Ca^{2+} ions. Quercetin decreased the depolarization-induced increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_C$), whereas it did not alter 4-AP-mediated depolarization and Na^+ influx. The quercetin-mediated inhibition of 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. Combined inhibition of protein kinase C (PKC) and protein kinase A (PKA) also prevented the inhibitory effect of quercetin on evoked glutamate release. Furthermore, quercetin decreased the 4-AP-induced phosphorylation of PKC and PKA. These results suggest that quercetin inhibits glutamate release from rat cortical synaptosomes and this effect is linked to a decrease in presynaptic voltage-dependent Ca^{2+} entry and to the suppression of PKC and PKA activity.

1. Introduction

Flavonoids are abundant components of the human diet and have recently received considerable attention due to their beneficial effects on the health, including prevention of cardiovascular disease, cancer, and neurodegenerative disease, as well as strengthening of the immune system (Middleton et al., 2000; Rossi et al., 2008; Echeverry et al., 2010). Quercetin, one of the most common compounds in the flavonoid family, is widely present in fruits and vegetables such as onions, broccoli, berries, and apples (Miean and Mohamed, 2001). Several biological activities of quercetin have emerged, for example, antihistamine, antiviral, antioxidant, anti-inflammatory, and anticarcinogenic properties (Zheng et al., 2005). In addition to these properties, quercetin has been confirmed to penetrate the blood–brain barrier (Youdim et al., 2004), attenuate oxidative stress-, oxygen-glucose deprivation- or neurotoxins-induced neurotoxicity (Ishige et al., 2001; Ha et al., 2003; Heo and Lee, 2004; Ansari et al., 2009; Zhang et al., 2011), and protect against ischemia-induced neuronal damage and cognitive dysfunction (Cho et al., 2006; Pu et al., 2007; Hwang et al., 2009; Yao et al., 2010). These findings suggest a neuroprotective role for quercetin; however, the mechanism whereby quercetin exerts this capability is not fully clarified.

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). Besides its physiological role, glutamate is also a potent neurotoxin and high levels in the synaptic cleft may lead to excitotoxicity. 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 (Meldrum, 2000). Accordingly, decreasing released glutamate is considered to be a potentially important mechanism for neuroprotective actions. Indeed, some neuroprotective agents have been revealed to decrease glutamate release in human and rat brain tissues (Wang et al., 2004; González et al., 2007). Quercetin has been observed to offer a neuroprotective effect and whether quercetin has an effect on endogenous glutamate release should be evaluated.

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To our knowledge, there are no studies addressing whether quercetin directly affects glutamate release at the presynaptic level. Thus, the purpose of the present study was to use isolated nerve terminals (synaptosomes) purified from the rat cerebral cortex to investigate the influence of quercetin on glutamate release. The experiments were performed with synaptosomes by monitoring the effects of quercetin on the release of endogenous glutamate, the synaptosomal plasma membrane potential, the Na^+ influx, and the activation of voltage-dependent Ca^{2+} channels (VDCCs). In addition, in view of the demonstrated role of various protein kinases in presynaptic modulation (Sihra and Pearson, 1995; Millan et al., 2003; Lin et al., 2010), this study also examined if the protein kinase signaling pathway participates in the regulation of glutamate release by quercetin.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats (100–200 g) were used in this study. All animal procedures were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Fu Jen Institutional Animal Care and Utilization Committee.

2.2. Chemicals

3′,3′,3′-Dipropylthiadicarbocyanine iodide [$DiSC_3(5)$], SBFI-AM, and fura-2-acetoxymethyl ester (Fura-2-AM) were obtained from Invitrogen (Carlsbad, CA, USA). ω-Conotoxin MVIIC (ω-CgTX MVIIC), dantrolene,7-chloro-5-(2-chloropheny)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP37157), 1-[N,O-bis(5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN62), 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059), N-[2-(p-bromocinnamylamino)-ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89), and bisindolylmaleimide I (GF109203X) were obtained from Tocris Cookson (Bristol, UK). Quercetin, 4-aminopyridine (4-AP), staurosporine, and all other reagents were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Quercetin, $DiSC_3(5)$, SBFI-AM, Fura-2, dantrolene, CGP37157, staurosporine, PD98059, and KN62 were dissolved in 0.1% dimethylsulfoxide (DMSO). 4-AP, and ω-CgTX MVIIC were dissolved in normal saline.

2.3. Synaptosomal preparation

Animals were killed by decapitation and cerebral cortex rapidly dissected. Purified synaptosomes were prepared by homogenizing the tissue in a medium that contained 320 mM sucrose, pH 7.4. The homogenate was spun for 2 min at 3000 × *g* (5000 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. 2 ml 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 the Bradford assay. The supernatants were discarded, and the synaptosomal pellets were stored on ice and used within 4–6 h.

2.4. 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 (McMahon and Nicholls, 1991). Synaptosomal pellets were resuspended in HBM that contained 16 μM BSA and incubated in a stirred and thermostatted cuvette maintained at 37 °C in a Perkin-Elmer LS-55 spectrofluorimeter (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). $NADP^+$ (2 mM), GDH (50 units/ml) and $CaCl_2$ (1.2 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. 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. In the absence of axonal connectivity, synaptosomes are not amenable to electrical stimulation, so several biochemical secretagogues have been developed, including the use of depolarization protocols involving potassium channel blockers such as 4-aminopyridine (4-AP) or high external KCl or direct mediation of Ca^{2+} entry using Ca^{2+}ionophores such as ionomycin (Barrie et al., 1991; Sihra et al., 1992; Nicholls, 1998). After a further 10 min of incubation, 4-AP (1 mM), KCl (15 mM), or ionomycin (5 μM) was added to stimulate glutamate release. Data were accumulated at 2-s intervals. A standard of exogenous glutamate (5 nmol) was added at the end of each experiment, and the fluorescence response used to calculate released glutamate was expressed as nanomoles glutamate per milligram synaptosomal protein (nmol/mg). Values quoted in the text and expressed in bar graphs represent levels of glutamate cumulatively released after 5 min of depolarization and are indicated as nmol/mg/5 min. Cumulative data were analyzed in Lotus 1-2-3 (IBM, White Plains, NY, USA) and MicroCal Origin (Origin Lab Corp., Northampton, MA, USA). Estimation of the IC_{50} was based on a one-site model [inhibition = (inhibition$_{MAX}$ × [quercetin])/(IC_{50} + [quercetin]) using the nonlinear curve-fitting function in MicroCal Origin.

2.5. Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_C$) measurement

$[Ca^{2+}]_C$ was measured using the Ca^{2+} indicator Fura-2-AM. Synaptosomes (0.5 mg/ml) were preincubated in HBM with 16 μM BSA in the presence of 5 μM Fura-2-AM and 0.1 mM $CaCl_2$, for 30 min at 37 °C in a stirred test tube. After Fura-2-AM loading, synaptosomes were centrifuged in a microcentrifuge for 30 s at 3000 × *g* (5000 rpm). The synaptosomal pellets were resuspended in HBM with BSA, and the synaptosomal suspension was stirred in a thermostated cuvette in a Perkin-Elmer LS-55 spectrofluorimeter (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). $CaCl_2$ (1.2 mM) was added after 3 min and further additions were made after an additional 10 min. 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, pH 7.5) to obtain minimum fluorescence in the absence of any Fura-2/Ca^{2+} complex. $[Ca^{2+}]_C$ was calculated