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Abstract

The role of cyclic ADP-ribose (cADPR) as a second messenger and modulator of the mTOR pathway downstream of dopamine (DA) receptors and/or CD38 was re-examined in the mouse. ADP-ribosyl activity was low in the membranes of neonates but was stimulated by DA *via* both D1- and D2-like receptors. ADP-ribosyl cyclase activity increased significantly during development in association with increased expression of CD38. The cADPR binding proteins, FKBP12 and FKBP12.6, were expressed in the adult mouse striatum. The ratio of phosphorylated to non-phosphorylated S6 kinase (S6K) in whole mouse striatum homogenates decreased after incubation of adult mouse striatum with extracellular cADPR for 5 minutes. This effect of cADPR was much weaker in MPTP-treated Parkinson's disease model mice. The inhibitory effects of cADPR and rapamycin were identical. These data suggest that cADPR is an endogenous inhibitor of the mTOR signaling pathway downstream of DA receptors in the mouse striatum and that cADPR plays a certain role in the brain in psychiatric and neurodegenerative diseases.

Key words: ADP-ribosyl cyclase, cyclic ADP-ribose, CD38, FKBP, S6K

Introduction

Dr. Toshiharu Nagatsu and the first author (H.H.) examined monoamine oxidase A and B (Nagatsu et al., 1981; Nakano et al., 1986a and b) and serotonin (Suzuki et al., 1983; Furuya et al., 10985) in rodent neuroblastoma clones, with permission from Dr. Nirenberg, National Institutes of Health, U.S.A. Dr. Nagatsu suggested that Higashida focused on amines and Parkinson's disease. However, Higashida's and his fellow researchers' main interests at that time were on bradykinin, muscarinic acetylcholine receptors and their coupling to phospholipase C (Yano et al., 1986; Higashida et al., 1986; Fukuda et al., 1988) and later to ADP-ribosyl cyclase (Higashida et al., 2001; Jin et al., 2007). Higashida and his colleagues concentrated on intracellular signaling leading to the modulation of membrane excitation and acetylcholine release (Hoshi et al., 2003). However, when Higashida and others used neuroblastoma cells as a tool to overexpress dopamine (DA) receptors (Higashida et al., 2013), they realized that there is little information on the coupling of DA receptors and ADP-ribosyl cyclase to a potential second messenger, cyclic ADP-ribose (cADPR), which is downstream of DA receptors.

DA receptors are involved in many physiological functions, such as

extrapyramidal motor control, short-term memory, attention, and reward (Greengard, 2001; Iversen and Iversen, 2007; Nagatsu, 2007; Nagatsu and Nagatsu, 2016). In contrast, abnormal activity of the DA system has been implicated in neurological and psychiatric disorders, such asParkinson's disease (PD), schizophrenia, bipolar disorder, and attention deficit hyperactivity disorder (Nagatsu, 2007). Therefore, the study of DA receptor-mediated intracellular signal transduction has been a primary approach to understanding the physiological functions or PD-related aspects of DA-related cellular responses (Baker et al., 2015).

From the viewpoint of signal transduction, D1- and D2-class DA receptor subtypes positively and negatively regulate adenylyl cyclase, respectively (Greengard, 2001; Missale et al., 1998). Stimulation of D2 receptors can increase intracellular Ca^{2+} concentrations by mobilizing Ca^{2+} from inositol-1,4,5-trisphosphate-sensitive stores (Frégeau et al., 2013). Increases in intracellular free calcium concentration ($[Ca^{2+}]_i$) seem to be mediated by the interaction of DA receptors with neuronal calcium sensor-1 or calcyon (Bergson et al., 2003). However, we recently reported a new pathway that is dependent on cyclic ADP-ribose (cADPR) (Lee, 2012) downstream of DA receptors and CD38 with ADP-ribosyl cyclase activity in rodents (Higashida et al., 2013).

cADPR is a co-factor of Ca^{2+}-induced Ca^{2+} release that activates Ca^{2+} release

from ryanodine receptors in microsomes (Lee, 2012; Hua et al., 1994; Okamoto et al., 2014; Higashida et al., 2001). cADPR is synthesized from β-NAD^+ by both cytosolic and membrane-bound forms of ADP-ribosyl cyclase and/or CD38 (Higashida et al., 2007; Kim, 2014). ADP-ribosyl cyclase activity increases upon stimulation of various receptors; some of this activity is observed in only neonates, not adult tissues of the same organs (Higashida et al., 2007).

The mammalian target of rapamycin (mTOR) pathway has emerged as a regulator of neuroplasticity in the central nervous system (CNS; Bockaert and Marin, 2015; Tramutola et al., 2016). mTOR is a Ser/Thr protein kinase complex that responds to multiple extracellular stimuli, such as nutrients, energy, growth factors, and mitogens that regulate cell growth, cell survival, transcription, and protein synthesis (Wullschleger et al., 2006; Hoeffer and Klann, 2010; Sukhbaatar et al., 2016). Deregulation of the mTOR pathways occurs in pathological conditions, such as cancer and neurodegenerative diseases characterized by long-term alterations in protein expression (Wullschleger et al., 2006). Administration of L-DOPA in a mouse model of Parkinson's disease leads to DA D1 receptor-mediated activation of mTOR complex 1 (mTORC1), which has been implicated in several forms of synaptic plasticity (Hoeffer and Klann, 2010). This response occurs selectively in GABAergic medium spiny

neurons that project directly from the striatum to the output structures of the basal ganglia. The L-DOPA-mediated activation of mTORC1 persists in mice that develop dyskinesia (Santini et al., 2009). Moreover, the mTORC1 inhibitor rapamycin prevents the development of dyskinesia without affecting the therapeutic efficacy of L-DOPA. Thus, the mTORC1 signaling cascade represents a promising target for therapeutics to treat the negative motor symptoms induced by anti-parkinsonian therapies (Santini et al., 2009; Lipton et al., 2014; Buszczak et al., 2014; Roohi and Hojjat-Farsangi, 2016).

mTORC1 is sensitive to rapamycin *via* competition between a mTOR regulatory protein (Raptor) and FKBP12-rapamycin for binding to the FRB domain (Buszczak et al., 2014; Thomson et al., 2009; Haeffer et al., 2008; Hausch, 2015). mTOR signaling is suppressed by rapamycin and FK506 in the brain. Removal of neuronal FKBP resulted in enhanced mTORC1 formation and increased phosphorylation of S6 kinase 1 (S6K1; Thompson et al., 2009). Thus, FKBP12 appears to repress mTORC1 activity. Another member of the FKBP family, FKBP12.6, can mediate the immunosuppressive effects of FK506 and act as a receptor for cADPR (Hoeffer et al., 2008; Hausch, 2015; Noguchi et al., 2007). The roles of FK12.6 and the FKBP12.6-cADPR complex were in part demonstrated by immunoblotting analysis of the striatum of control and MPTP-treated mice (Higashida et al., 2013). Here, we

re-examined the mouse striatum to support our hypothesis that cADPR functions as an endogenous modulator of the mTOR pathway downstream of DA receptors.

Materials and Methods

Membrane preparation

Crude membrane fractions were prepared as described previously from male ICR mice for the ADP-ribosyl cyclase assays (Higashida et al., 1997).

Fluorometric measurement of ADP-ribosyl cyclase

ADP-ribosyl cyclase activity was determined fluorometrically using a technique based on measuring the conversion of β-NGD^+ into the fluorescent product cyclic GDP-ribose (cGDP-ribose), as described previously (Higashida et al., 1997; Higashida et al., 2002; Greff and Lee, 2002). The samples were then excited at 300 nm, and fluorescence emission was monitored continuously at 410 nm with a spectrofluorophotometer (RF-6000; Shimadzu, Kyoto, Japan).

Incubation of the striatum

Striata were isolated from the brains of 9-week-old male ICR mice or mice treated with

an intraperitoneal injection of MPTP (20 mg/kg, 4 times/day at 2-hour intervals; Higashida et al., 2013). The striata were kept in medium (pH 7.3) containing 124 mM NaCl, 5 mM KCl, 1.24 mM KH_2PO_4, 2 mM $CaCl_2$, 25.9 mM $NaHCO_3$, and 10 mM glucose for 1 hour at room temperature and bubbled with a mixture of 95% O_2/5% CO_2. The tissue was further incubated with or without 100 μM cADPR, 100 nM DA or 100 nM DA + 100 μM cADPR for 5 minutes at 32°C. Certain tissues were preincubated with 8-Bromo-cADPR (Sigma-Aldrich Sweden AB, Stockholm, Sweden) for 1 hour. Then, tissues were immediately homogenized in lysis buffer containing 0.1% Triton X-100, 10% glycerol, 1.5 mM EDTA, 0.5 mM Na_3VO_4, 10 mM NaF, and 1× protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration was determined by BCA assay (Thermo Fisher Scientific, Inc., Waltham, MA).

Western blotting and PCR

Aliquots of 30 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The membranes were incubated with anti-phospho-p70 S6K (Thr389) and anti-p70 S6K antibodies (both from Cell Signaling Technology, Beverly, MA) in blocking buffer overnight at 4°C and then for 3 hours at room temperature (both antibodies were diluted 1:500). The membranes were then processed and visualized as

described previously (Higashida et al., 2013). RT-PCR analysis was performed as described previously (Higashida et al., 2013).

Statistics

All results are expressed as the mean ± SEM. One- or two-tailed Student's t-tests and one-way ANOVA combined with the Bonferroni test were used to analyze data with unequal variance between groups. Two-way ANOVA was used to assess *Treatment* x *Concentration* interaction. In all analyses, $P < 0.05$ was considered to indicate statistical significance.

Results

ADP-ribosyl cyclase activity in the mouse striatum

ADP-ribosyl cyclase activity was measured in crude membrane fractions isolated from the striatum of mice at various ages. Basal ADP-ribosyl cyclase activity was very low, *i.e.*, approximately 0.1 to 1.6 pmol cGDPR formed/min/mg protein in preparations from 1–3 day prenatal and 1–5 day postnatal mice, respectively. ADP-ribosyl cyclase activity increased sharply with age after postnatal day 5 (Figure 1; $P < 0.001$, $F_{(12,65)} = 59.68$, $R^2 = 0.9168$, one-way ANOVA), reaching a very high level by 12 days of age (21.6 ± 3.7

pmol cGDPR formed/min/mg protein, $n = 12$), which was 40-fold higher than the basal activity at birth ($P < 0.001$, Bonferroni's *post-hoc* test).

Sensitivity to dopamine

ADP-ribosyl cyclase activity was analyzed in striatal membranes from mouse neonates at birth (day 0) and postnatal days 1–11 in the presence of 100 nM DA in the reaction mixture. From neonatal day 1 to 4, the increase ranged from 130% to 310%. However, after 12 days, DA-induced activation dropped to less than 105 ± 10% and remained at this level until the adult stage.

The concentration–response relationship to DA was examined (Figure 2). The increases induced by DA (with or without 10 nM GTP), SKF38393 (SKF, a D1-like agonist), and bromocriptine mesylate (BC, a D2-like agonist) were dose-dependent. Two-way ANOVA demonstrated significant *Treatment (±GTP)* x *Concentration (DA)* interaction ($P = 0.0261$, $F_{(6,126)} = 2,49$; Figure 2A). One-way ANOVA demonstrated significant difference in experiments with SKF ($P < 0.001$, $F_{(5,54)} = 12.88$, $R^2 = 0.5439$) and BC treatment ($P < 0.001$, $F_{(4,45)} = 17.00$, $R^2 = 0.6018$; Figure 2B). The maximum effects of these three agents were obtained at different concentrations between 1 and 100 nM. The maximum response of 202.8 ± 21.4% of the control ($n = 10$, $P < 0.01$,

Bonferroni's *post-hoc* test) was obtained with 100 nM DA. Significant increases to 183.0 ± 12.2% ($n = 10$, $P < 0.01$, Bonferroni's *post-hoc* test) and 157.9 ± 5.3% ($n = 10$, $P < 0.01$) of the control were obtained with 10 nM SKF and 1 nM BC, respectively.

CD38 expression

We examined whether the increased ADP-ribosyl cyclase activity is due to an increase in CD38 expression. While a band of approximately 42 kDa was detected in the 1-week striatum, CD38 protein abundance was significantly increased after 2 weeks and in adult mice (10 weeks old), as shown by Western blotting (Figure 3).

RT-qPCR analysis indicated that CD38 mRNA expression levels increased significantly with age (data not shown). CD38 mRNA expression relative to β-actin expression was approximately 29.8-fold higher in adult mice (2 weeks old) than in mice at postnatal day 1 ($P < 0.001$, two-tailed *t* test).

Expression of FKBP in the mouse brain

Next, the expression of FKBP12 and the close homolog FKBP12.6 was examined in various brain regions of 9-week-old male mice by RT-PCR. FKBP12 was ubiquitously detected in four regions (cerebrum, cerebellum, hypothalamus and striatum) and the

pituitary, with no significant differences among them, based on the relative expression normalized to β-actin (Higashida et al., 2013). In contrast, the FKBP12.6 expression level was lower in the striatum than in the cerebrum, cerebellum, and hypothalamus in 9-week-old male mice (Figure 4), suggesting region-specific expression. The result provides minimal necessary evidence for a functional role of cADPR/FKBP12 or cADPR/FKBP12.6 binding complexes in mTOR signaling in the striatum.

Effects of cADPR on S6 kinase

The mammalian target of rapamycin (mTOR) activates S6 kinase (S6K), which is responsible for phosphorylation of the ribosomal protein S6 (S6), a component of the 40S ribosomal subunit (Bockaert and Marin, 2015; Busxczak et al., 2014). cADPR (100 μM) was added to the incubation medium for 9-week-old mouse striatum samples for 5 minutes with or without DA receptor stimulation by 100 nM DA (Figure 5A and B). DA itself did not cause significant inhibition of S6K phosphorylation, being calculated as the percentage change between 0 and 5 minutes (P-S6K/S6K at 5 minutes divided by P-S6K/S6K at 0 minutes): 1.28 ± 0.16 (n = 22; Table 1) in the absence and 1.42 ± 0.25 (n = 5) in the presence of DA. This result well accords with the report in the nucleus accumbens by D1 stimulation (Sutton and Caron, 2015).

One-way ANOVA demonstrated no significant difference between treatments without (Figure 5A; $P > 0.05$, $F_{(2,43)} = 2.595$, $R^2 = 0.1077$) or with DA receptor stimulation (Figure 5B; $P = 0.2741$, $F_{(2,45)} = 1.332$, $R^2 = 0.0558$). In whole-cell homogenates, S6K phosphorylation at Thr389 significantly decreased in both the presence and absence of DA ($n = 16$ mice each, $P < 0.01$, Bonferroni's *post-hoc* test)). This cADPR-induced decrease in S6K phosphorylation was reversed by prior administration of 100 μM 8-bromo-cADPR, a cADPR antagonist ($n = 14$, $P < 0.02$, Bonferroni's *post-hoc* test)). The total amount of S6K was unaffected by cADPR treatment with or without DA or 8-bromo-cADPR.

The effects of cADPR were compared with those of rapamycin. Rapamycin was added to the incubation medium 1 h prior to experiments at a final concentration of 100 μM (Table 1). One-way ANOVA demonstrated significant effects of antagonist treatment ($P < 0.0001$, $F_{(2,53)} = 4152$, $R_2 = 0.9937$). Significant inhibition (44% of that without rapamycin) of S6K phosphorylation at Thr389 in whole-cell homogenates was obtained with rapamycin ($n = 16$, $P < 0.001$, Bonferroni's *post-hoc* test), which was equivalent to the effect of cADPR (59%). In addition, we determined that the decreased kinase activity is independent of protein kinase C but slightly influenced by protein kinase A (Higashida et al., 2013).

Discussion

The results of the present study, together with our previous report (Higashida et al., 2013), indicate that ADP-ribosyl cyclase activity in the mouse striatum is enhanced by DA stimulation during the neonatal period (up to about postnatal day 11) but not in adults. These observations agree with those of our previous study of angiotensin II receptors in ventral cardiac cells (Higashida et al., 2000), in which activation by angiotensin was observed on neonatal day 4. Similar activation of ADP-ribosyl cyclase by Gs-coupled D1-like receptors and Gi-coupled D2-like receptors was obtained in the mouse striatum. However, this is not unexpected, because we have shown that Gi-coupled mGluR3 (type IIa) stimulates ADP-ribosyl cyclase, while Gs-coupled mGluR1 and mGluR5 (type Ia) also stimulate ADP-ribosyl cyclase (Higashida et al., 2003). Therefore, DA may play a certain role in the early development through cADPR.

In the adult period, ADP-ribosyl cyclase activity was > 40-fold higher in the mouse (current results) and > 100-fold higher in the rat (Higashida et al., 2013). The mRNA and protein levels of CD38 were significantly increased after postnatal days 5 – 10. It is possible that cADPR is abundant as an endogenous intermediate product of ADP-ribosyl cyclase and/or CD38 in the adult mouse striatum.

cADPR significantly inhibited the phosphorylation of S6K, the translational regulatory kinase and downstream target of mTORC1 (Bockaert and Marin, 2015; Wullschleger et al., 2006; Hoeffer et al., 2010). This cADPR-induced inhibition of S6K phosphorylation was reproduced by rapamycin, a well-known inhibitor of mTOR signaling through FKBP (Lipton and Sahin, 2014; Hoeffer et al., 2008). This result seems to indicate that cADPR shares the well-documented rapamycin pathway (Bockaert and Marin, 2015; Wullschleger et al., 2006; Lipton and Sahin, 2014; Buszczak et al., 2014). Therefore, since exogenous cADPR and rapamycin inhibited the mTOR pathway, it is possible that endogenously synthesized cADPR functions to inhibit mTOR signaling in the intact adult striatum (Figure 6).

Interestingly, with regard to PD, we have shown that cADPR-induced S6K phosphorylation was diminished in the MPTP-treated mouse brain (see also Higashida et al., 2013). This observation is consistent with the enhanced mTOR signaling in the PD model brain (Bockaert and Marin, 2015). It is hypothesized that when the mTOR signal is enhanced by the chronic use of L-DOPA, translation is subsequently increased, which may lead to involuntary movement (Santini et al., 2009; Lipton and Sahin, 2014).

In sum, we propose that the endogenous presence of cADPR is likely a safeguard for maintaining or suppressing translation at a constant rate under conditions

of mTOR activation observed in brain dysfunction, such as that in PD (Figure 6).

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Table 1 Effects of cADPR and rapamycin on S6 kinase phosphorylation in the male mouse striatum

Inhibitors	Ratio	%	*n*	*P*
None	1.28 ± 0.16	(100)	22	
cADPR	0.76 ± 0.04	(59)	18	< 0.05
Rapamycin	0.56 ± 0.03	(44)	16	< 0.02

After adaptive preincubation for 60 minutes, striatal slices were incubated with or without 100 μM cADPR or 100 μM rapamycin for 5 minutes at 35°C. Changes in S6K phosphorylation were quantified after a 5-minute incubation (P-S6K/S6K). Data were alculated as the percentage change between 0 and 5 minutes (P-S6K/S6K at 5 minutes divided by P-S6K/S6K at 0 minutes). Data are presented as the mean ± SEM of 16–22 preparations. *P*, relative to control (two-tailed *t* test).

Table 2. Effects of cADPR on S6 kinase phosphorylation in striata isolated from MPTP-treated mice

	Ratio (%)	Ratio (%)
Mice	Control	MPTP-treated
None	1.19 ± 0.13 (100)	1.22 ± 3.4 (100)
+cADPR	0.80 ± 0.09 (67)*	0.93 ± 0.16 (76)$^{n.s.}$

After adaptive preincubation for 60 minutes, striatal slices were incubated with or without 100 μM cADPR for 5 minutes at 35°C. Changes in S6K phosphorylation were quantified after a 5-minute incubation (P-S6K/S6K). Data were calculated as the percentage change between 0 and 5 minutes (P-S6K/S6K at 5 minutes divided by P-S6K/S6K at 0 minutes). Data are presented as the mean ± SEM. N = 14 (none) and N = 16 (+cADPR). $^{*}P < 0.02$ relative to control (two-tailed *t* test).

FIGURE LEGENDS

Figure 1. Developmental regulation of basal ADP-ribosyl cyclase activity in striatal membranes of mice at various ages. Developmental time course of cyclase activity. ADP-ribosyl cyclase was measured fluorometrically as the rate of cGDPR formation. Data are presented as the mean ± SEM of 4–6 determinations. *, Significantly different from postnatal day 1 ($P < 0.001$, Bonferroni's *post-hoc* test). One unit represents 20.5 pmol/min/mg protein.

Figure 2. Relationship between ADP-ribosyl cyclase activity and various concentrations of DA or two DA agonists with or without GTP in the reaction mixture.

Cell membranes were isolated from the striata of 3-day-old neonatal mice. (**A**) Relationship between cGDPR formation and DA concentration with (closed circles) or without 10 nM GTP (open circles). (**B**) Concentration dependency of SKF (squares) and BC (circles) on cGDPR formation. $n = 10$. Two-way (**A**) or one-way (**B**) ANOVA with *post-hoc* Bonferroni's test was evaluated. * and **, Significantly different from control ($P < 0.05$ and $P < 0.001$, respectively, one-way ANOVA).

Figure 3. Representative results of Western blotting analysis of mouse CD38. Cell lysates from the striata of mice at the indicated ages were separated by 8% SDS-PAGE. The blot was probed with an anti-murine CD38 antibody.

Figure 4. FKBP12.6 mRNA expression. FKBP12.6 mRNA expression was analyzed by RT-PCR in four brain regions and the pituitary in 9-week-old male mice using β-actin mRNA as an internal control. Quantitative data are shown as the mean ± SEM (n = 5 independent experiments) of FKBP12.6/actin. One-way ANOVA followed by Bonferroni's post hoc test: $F_{4,12} = 30.99$, $^{*}P < 0.002$, $^{**}P < 0.001$. Modified from Figure 6 of our previous report [9].

Figure 5. Effects of cADPR on S6 kinase phosphorylation in the striata of 9-week-old male mice. After adaptive preincubation for 60 minutes, striatal slices were incubated with or without 100 μM cADPR alone or together with 100 μM 8-bromo-cADPR (8-Br-cADPR) for 5 minutes at 35°C. Representative Western blots of phosphorylated S6 kinase (Thr389; P-S6K) and total S6 kinase (S6K) in the absence (**A**) or presence (**B**) of 100 nM DA. Changes in S6K phosphorylation were quantified after a

5-minute incubation (P-S6K/S6K). Data were calculated as the percentage change between 0 and 5 minutes (P-S6K/S6K at 5 minutes divided by P-S6K/S6K at 0 minutes). Data are presented as the mean ± SEM of 16 controls (without cADPR and 8-Br-cADPR), 22 preparations treated with cADPR, and 8 samples treated with cADPR+8-Br-cADPR. One-way ANOVA with Bonferroni's *post-hoc* test was evaluated for significance (see text). $^{*}P < 0.03$ for the comparison of control and 8-Br-cADPR (two-tailed *t* test). Modified from Figure 6 of our previous report [9].

Figure 6. Schematic of the proposed role of endogenous cADPR and exogenous rapamycin in the striatum. cADPR or rapamycin with FKBP inhibits the mTORC1 pathway, in which S6 is phosphorylated by S6K.

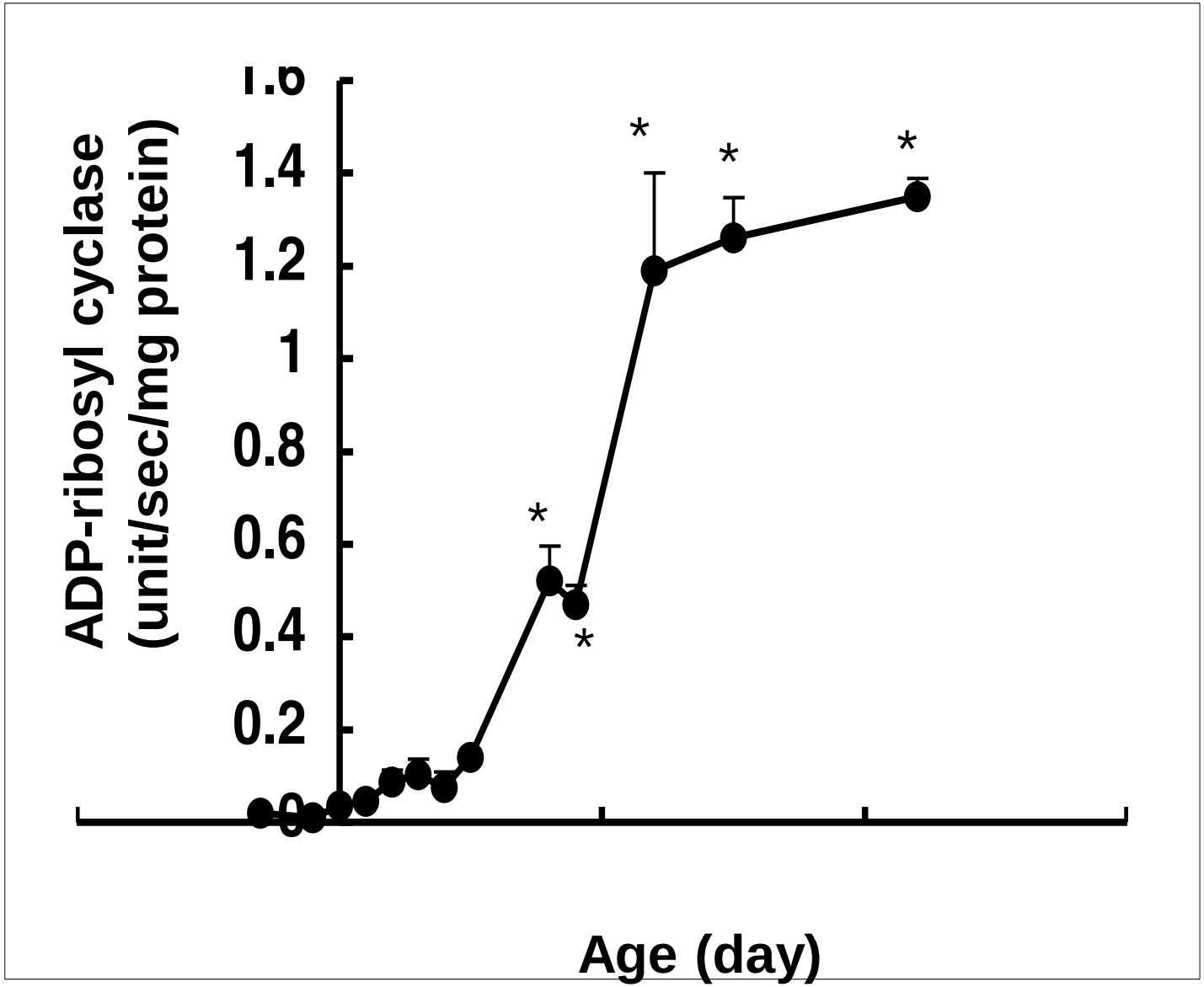

ADP-ribosyl cyclase
(unit/sec/mg protein)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
*
*
Age (day)

Fig. 1

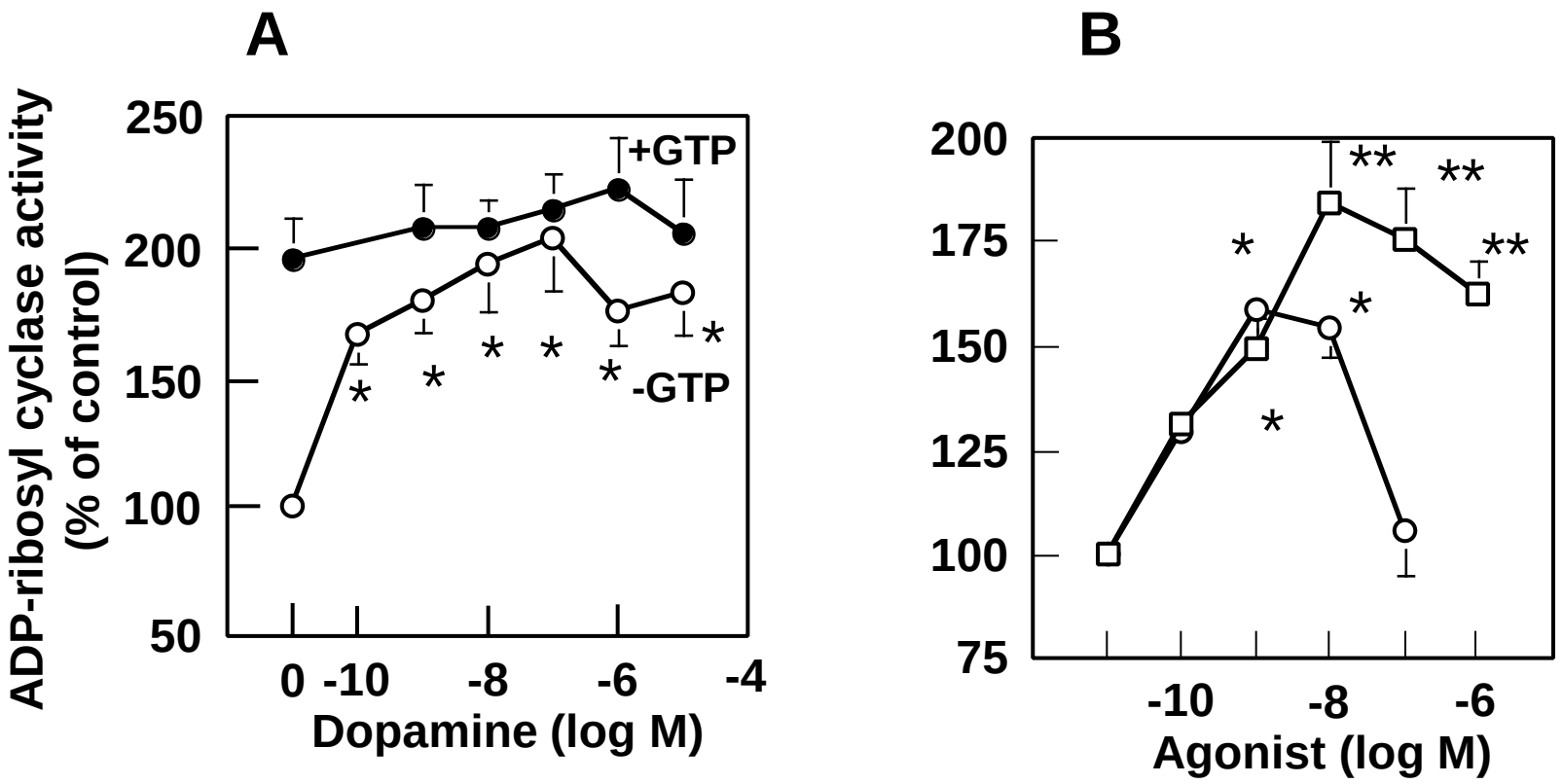

A
ADP-ribosyl cyclase activity
(% of control)
250
200
150
100
50
+GTP
-GTP
*
0
-10
-8
-6
-4
Dopamine (log M)
B
200
175
150
125
100
75
**
*
-10
-8
-6
Agonist (log M)

Fig. 2

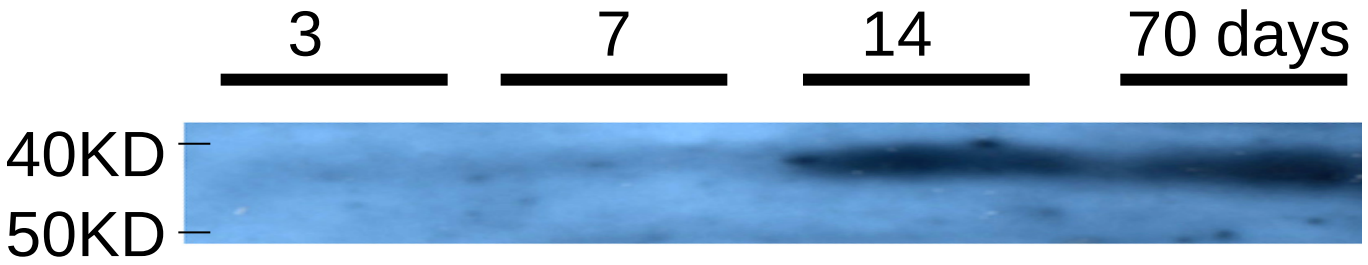


Fig. 3

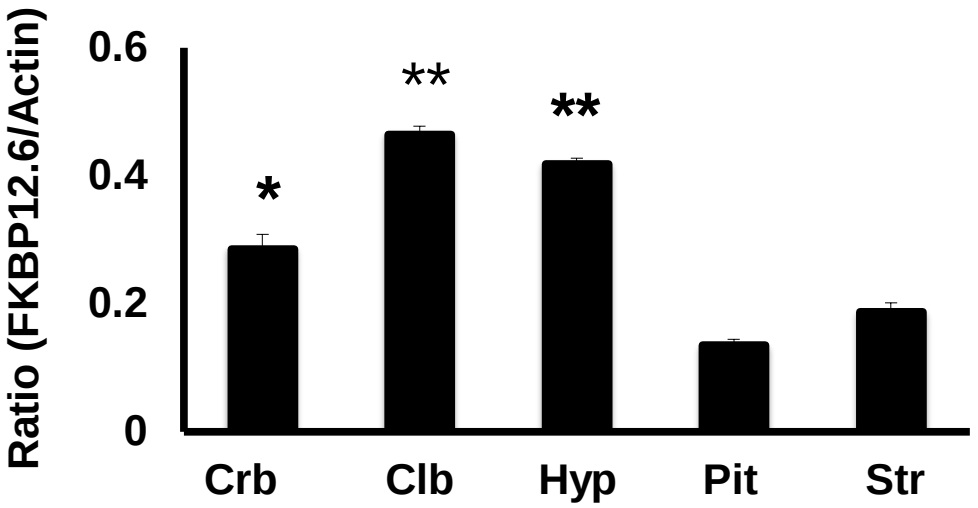


Fig. 4

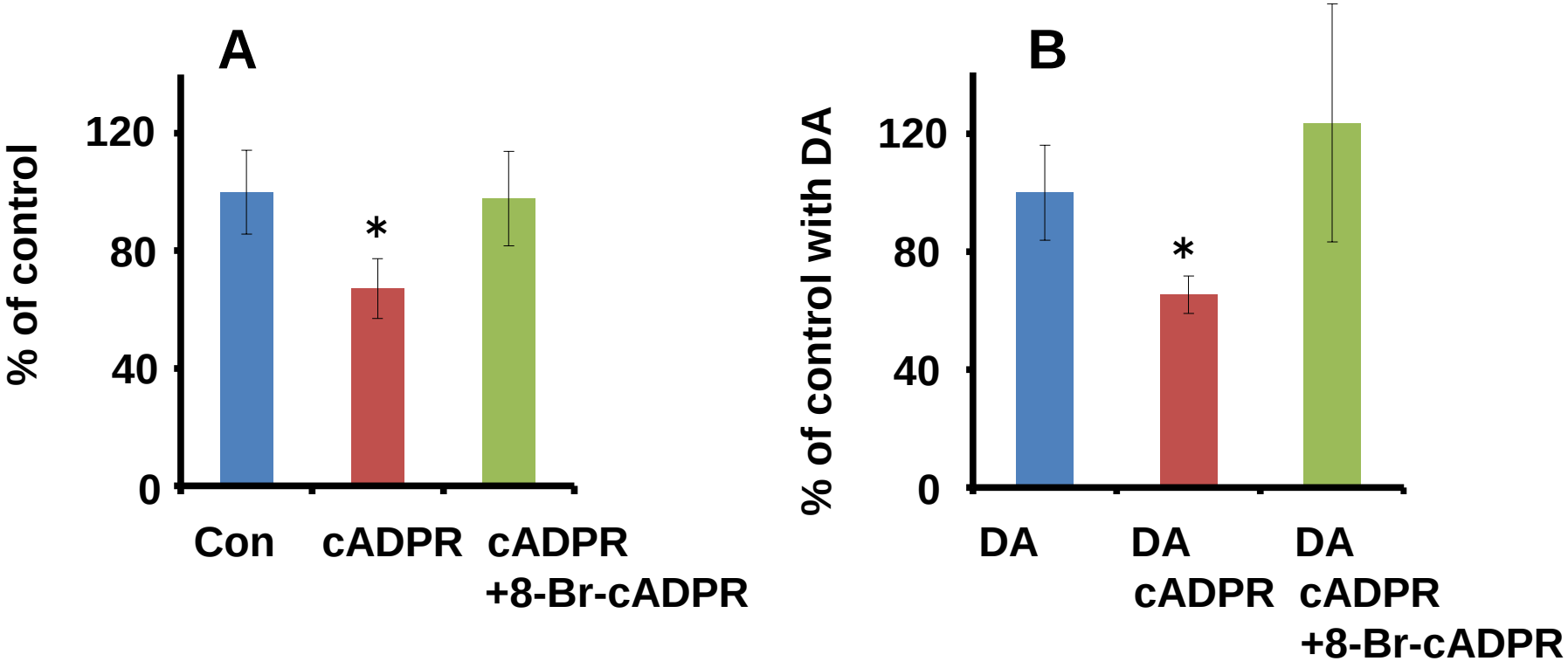
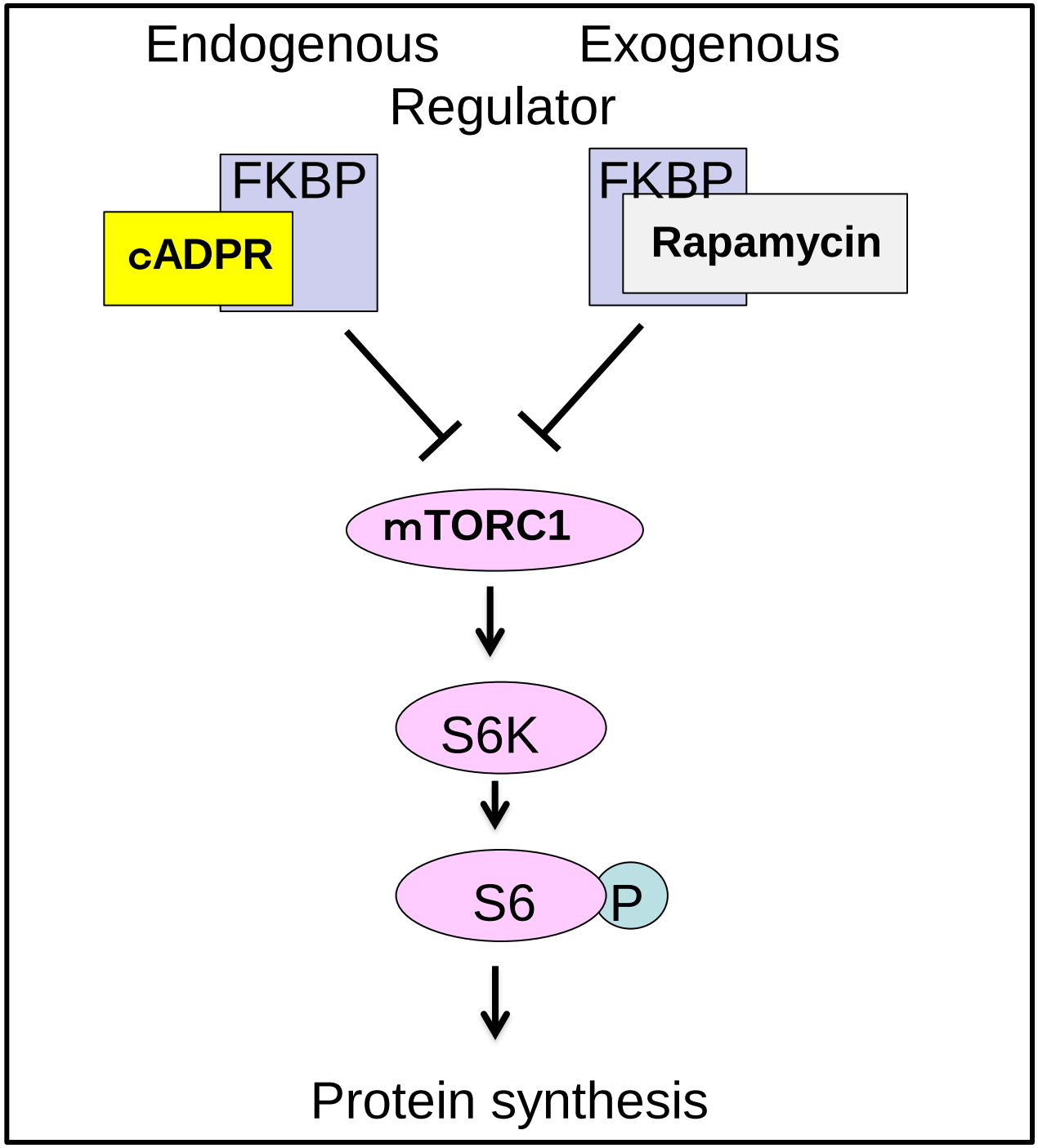

A
% of control
120
80
40
0
*
Con
cADPR
cADPR
+8-Br-cADPR
B
% of control with DA
120
80
40
0
*
DA
DA
cADPR
DA
cADPR
+8-Br-cADPR

Fig. 5


Endogenous
Exogenous
Regulator
FKBP
cADPR
FKBP
Rapamycin
mTORC1
S6K
S6
P
Protein synthesis