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Short communication

Ryanodine receptor modulation by diadenosine polyphosphates in synaptosomal and microsomal preparations of rat brain

P. Nickolas Shepel[a,b], Clark P. Holden[a], Jonathan D. Geiger[a,b,*]

[a] *Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada R2H 2A6*
[b] *Division of Neurovirology and Neurodegenerative Disorders, St. Boniface Research Center, R4046-351 Tache Avenue, Winnipeg, Manitoba, Canada R2H 2A6*

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Abstract

Diadenosine polyphosphates (Ap_nAs) are transmitter-like substances that act intracellularly via unclear mechanisms. Here we tested hypotheses that diadenosine tetraphosphate (Ap_4A) modulates ryanodine binding in microsomal and synaptosomal fractions of rat brain, and that Ap_4A affects modulation of ryanodine binding by divalent cations and caffeine. Using [^{3}H]ryanodine-binding assays, we showed that Ap_4A produced significant and concentration-dependent increases in [^{3}H]ryanodine binding in microsomes and these actions were reduced by Mg^{2+} and potentiated by caffeine. In synaptosomal subfractions, effects of Ap_4A on [^{3}H]ryanodine binding were most profound in subfractions enriched in synaptic vesicle-associated protein synaptophysin. These results suggest that Ap_nAs and ryanodine receptors are well placed to modulate Ca^{2+}-dependent synaptic processes.

Keywords: Synaptophysin; Dinucleotide oxidized; GRP-78; Subcellular fractionation

1. Introduction

In many tissues, including neurons, levels of intracellular calcium ($[Ca^{2+}]_i$) are regulated, at least in part, by ryanodine receptor-regulated Ca^{2+} release channel complexes that upon activation can facilitate Ca^{2+}-induced Ca^{2+} release (Pozzan et al., 1994). Ryanodine receptors are heterogeneously distributed in the brain (Padua et al., 1991; Stein et al., 1992), are found in nerve terminals where they contribute in Ca^{2+}-induced neurotransmitter release (Llano et al., 2000), and may be localized in specialized endoplasmic reticulum subcompartments or distinct subcellular organelles (Padua et al., 1996).

Ryanodine receptor binding and function are regulated by adenine nucleotides, caffeine, and divalent cations (Sitsapesan et al., 1995). Our interest in ryanodine receptor modulators is focused on diadenosine polyphosphates (Ap_nAs; $n=2-6$), a group of endogenous, physiologically active compounds that have a relatively strong modulatory effect on ryanodine receptors compared to other adenine nucleotides (Holden et al., 1996). Ap_nAs are formed during the amino acid activation step in protein synthesis (Kisselev and Wolfson, 1994; Zamecnik et al., 1966), and can function as neurotransmitters (Miras-Portugal et al., 1998) because they are released in a neurotransmitter-like fashion (Pintor et al., 1992, 1993), are metabolized by ectohydrolase enzymes (Rodriguez-Pascual et al., 1992), and interact with extracellular P_{2X} (Tepel et al., 1996), P_{2D} (Pintor and Miras-Portugal, 1995a), and dinucleotide (P_4) receptors on presynaptic terminals (Diaz-Hernandez et al., 2001; Pintor and Miras-Portugal, 1995b). The intracellular actions of Ap_nAs are not well understood; however, intracellular levels increase during periods of cell proliferation (Rapaport and Zamecnik, 1976; Vartanian et al., 1997; Weinmann-Dorsch et al., 1984) and under conditions of cell stress (Baker and Jacobson, 1986; Jovanovic et al., 1998; Lee et al., 1983).

Results from previous studies showing [^{3}H]ryanodine-binding activity in synaptosomal brain fractions (Padua et al., 1996), that $[Ca^{2+}]_i$ released from ryanodine-sensitive stores in the synaptic region alters neurotransmitter signaling (Llano et al., 2000) and synaptic plasticity (Balschun et al., 1999; Emptage et al., 2001), and that Ap_nAs are stored

* Corresponding author. Division of Neurovirology and Neurodegenerative Disorders, St. Boniface Research Center, R4046-351 Tache Avenue, Winnipeg, Manitoba, Canada R2H 2A6.
E-mail address: Geiger@cc.umanitoba.ca (J.D. Geiger).

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in synaptic vesicles (Pintor et al., 1992) prompted us to investigate the relationship between ryanodine receptors and synaptic vesicles as well as the mechanism by which Ap_nAs may alter the function of these receptors. Here we tested hypotheses that ryanodine receptors are associated with synaptic vesicles, that Ap_4A modulates ryanodine binding in vesicular fractions, and that Ap_4A affects modulation of ryanodine binding by other agents such as divalent cations and caffeine. Using [^{3}H]ryanodine-binding assays to assess the functional state of the ryanodine receptor-regulated $[Ca^{2+}]_i$ release channel, as well as subcellular fractionation and immunoblot techniques, we showed that Ap_nA interactions with the ryanodine receptor act to prolong the Ca^{2+}-activated state of the receptor and that this effect, although very evident in microsomal fractions, was even more pronounced in synaptic subfractions enriched in synaptophysin protein.

2. Materials and methods

2.1. Animals

Adult male Sprague–Dawley rats obtained from the University of Manitoba Central Animal Care breeding facility weighing 200 ± 20 g were used in these studies. All protocols were performed in accordance with University of Manitoba Animal Care Ethics Committee guidelines and Canada Council on Animal Care regulations.

2.2. Chemicals

[9,21-^{3}H(*N*)]Ryanodine (specific activity of 60 Ci/mmol) was purchased from Dupont-New England Nuclear; unlabeled ryanodine was obtained from Research Biochemicals. The sodium salt of periodate-oxidized P^1,P^4-di(adenosine-5′)tetraphosphate (oAp_4A) and ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were obtained from Sigma (St. Louis, MO).

2.3. Tissue preparation

Tissue fractions were prepared essentially as previously described (Padua et al., 1996). Brains from decapitated rats were homogenized in 20 ml of cold buffer A composed of 0.32 M sucrose, 5.0 mM piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid)-KOH (PIPES-KOH), and 1.0 mM phenylmethylsulfonyl fluoride (PMSF) with pH adjusted to 8.0. Homogenate was centrifuged at $10{,}000 \times g$ for 30 min at 4 °C and the resulting supernatant was centrifuged at $100{,}000 \times g$ for 70 min to yield a microsomal (P3) pellet. P3 pellets were suspended in 2.0 ml of buffer B containing 20 mM PIPES-KOH, 1.2 mM $MgCl_2$, 200 mM KCl, 1.0 mM PMSF, and 100 μM $CaCl_2$, giving final total protein concentrations between 2.3 and 5.0 mg/ml. For experiments involving divalent cations and caffeine, 0.2 mM EGTA (final concentration) was used in P3 preparations.

Synaptosomal subfractions were prepared as described previously (Padua et al., 1996). Following decapitation, cerebrums from two rats were dissected and homogenized in buffer A (10 ml/g tissue). Homogenate was centrifuged at $1000 \times g$ for 10 min and the supernatant was centrifuged at $12{,}000 \times g$ for 20 min to yield a synaptosomal (P2′) pellet which was then suspended to 2.0 ml/g of original tissue mass in 5.0 mM HEPES-NaOH (pH 7.4). P2′ homogenates, except for aliquots saved for binding and immunoblot analysis, were centrifuged at $10{,}000 \times g$ for 30 min and the supernatants were loaded onto a discontinuous sucrose gradient consisting of 0.4, 0.6, and 0.8 M sucrose in 5.0 mM HEPES-NaOH (pH 7.4) buffer. Gradients were centrifuged at $53{,}000 \times g$ for 2 h and synaptosomal subfractions collected were as follows; layer above the 0/0.4 M interface (fraction 1), 0/0.4 M interface (fraction 2), 0.4 M layer (fraction 3), 0.4/0.6 M interface (fraction 4), 0.6/0.8 M interface (fraction 5), and pellet (fraction 6). Subfractions were diluted 1:4 with buffer B, centrifuged at $100{,}000 \times g$ for 2 h, and pellets were suspended in 500 μl of buffer B.

2.4. Ryanodine receptor binding

Binding assays were conducted in duplicate as described previously (Padua et al., 1994) using a final assay volume of 250 μl. All [^{3}H]ryanodine-binding assays, except those measuring divalent cation/caffeine sensitivity, were conducted in a low ionic strength magnesium buffer (buffer B). Divalent cations and caffeine were added to Ca^{2+}/Mg^{2+}-free buffer B to their desired final concentrations as calculated using a software program (Brooks and Storey, 1992). Unless indicated otherwise, the concentration of oAp_4A used was 111 μM. Total protein concentrations were determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin standards. Data were reported as mean ± S.E.M. with statistical calculations carried out using INSTAT2 (Biosoft). Statistical significance was determined using a one-way analysis of variance (ANOVA) with Student–Newman–Keuls multiple comparisons test.

2.5. Immunoblotting

Synaptosomal subfractions extracted from sucrose gradients were diluted 1:4 with lysis buffer containing 5 mM Tris–HCl, 0.5 mM $MgCl_2$, 0.5 mM PMSF, 2.5 mM dithiothreitol, 1 mM benzamidine, 2 μg/ml pepstatin A, 1 μg/ml aprotinin (pH 7.0), centrifuged at $100{,}000 \times g$ for 2 h, and pellets were immediately frozen at −80 °C until used for analysis. Total protein in samples was quantified using a commercial kit from Bio-Rad (Richmond, CA), separated by polyacrylamide gel electrophoresis (10% gels, 150 V, 90 min), and then immobilized onto a nitrocellulose support (Osmonics, Westborough, MA) by semi-dry trans-

fer (15 V, 30 min) using a cold transfer buffer containing 48 mM Tris-base, 39 mM glycine, 20% methanol, and 0.038% sodium dodecyl sulfate (SDS) adjusted to pH 8.3. Monoclonal mouse anti-synaptophysin (SVP-38, Sigma) and polyclonal goat anti-GRP-78 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used in 1:5000 and 1:2000 dilutions, respectively. Peroxidase-conjugated rabbit anti-goat and goat anti-mouse secondary antibodies (Jackson Immuno Research Labs, West Grove, PA) were used in 1:5000 and 1:10,000 dilutions, respectively. Primary and secondary antibodies were incubated with nitrocellulose membranes for 2 h and 30 min, respectively, at room temperature. Blocking buffers (diluted with Tris-buffered saline, TBS) and chemiluminescence reagents were supplied in a commercial blotting kit (Boehringer Mannheim).

3. Results

Caffeine, divalent cation, and adenine nucleotide modulation of ryanodine binding are best observed when binding assays are conducted in low ionic strength buffers where binding levels are uniformly lower than when high ionic strength buffers are used (Padua et al., 1994). Here we observed, using low ionic strength conditions in the presence of 1.2 mM $MgCl_2$ and 100 μM $CaCl_2$, [^{3}H]ryanodine binding levels between 5.9 ± 0.4 and 6.8 ± 1.6 fmol/mg protein in microsomal fractions (Fig. 1) and higher levels ranging from 10.1 ± 2.6 to 27.9 ± 6.5 fmol/mg protein in synaptosomal subfractions (Fig. 2).

[^{3}H]Ryanodine binding in microsomal fractions was enhanced significantly ($p < 0.05–0.001$) by oAp_4A in a concentration-dependent fashion (Fig. 1A). At 111 μM oAp_4A, statistically significant [^{3}H]ryanodine binding enhancement was observed at 0.1 μM Ca^{2+} (1.7-fold) and reached a maximum (7.4-fold) at 100 μM Ca^{2+} (Fig. 1B).

At concentrations of free Ca^{2+} ranging from 0.1 to 1000 μM, magnesium attenuated and caffeine potentiated oAp_4A-induced increases in [^{3}H]ryanodine binding (Fig. 1C). Beginning at 0.1 μM Ca^{2+}, 5.0 mM Mg^{2+} consistently reduced [^{3}H]ryanodine binding to approximately one-half of the control levels (1.2 mM Mg^{2+}). Caffeine, in contrast, significantly potentiated oAp_4A-induced increases in [^{3}H]ryanodine binding at Ca^{2+} concentrations ranging from 0.1 to 1000 μM (Fig. 1C).

Given our previous observations that [^{3}H]ryanodine-binding sites were present in synaptosomal subfractions of rat brain (Padua et al., 1996), we investigated whether RyRs in these subfractions were sensitive to the actions of Ap_nAs (Fig. 2A). Immunoblot analyses using antibodies to GRP-78 (ER marker) or synaptophysin (synaptic vesicle marker, SVP-38) demonstrated no detectable GRP-78 signal in subfraction 1, but GRP-78 in increasing abundance in subfractions 2 to 6 (Fig. 2B). In contrast, we observed consistently very high levels of synaptophysin in subfraction 1 and a general reduction in synaptophysin signal in fractions 2 through 6. Compared to the crude synaptosomal P2′ fraction, subfraction 1 was highly enriched in synaptophysin and presumably synaptic vesicles. In decreasing order of enrichment in synaptic vesicles (Whittaker et al., 1964), [^{3}H]ryanodine binding was 14.5 ± 1.3 in subfraction 1, 27.8 ± 6.5 in subfraction 2, 25.9 ± 7.3 in subfraction 3, and 18.8 ± 2.6 fmol/mg protein in subfraction 4 (Fig. 2A). oAp_4A (111 μM) significantly ($p < 0.01$) increased [^{3}H]ryanodine binding in subfraction 1 by 600%, subfraction 2 by 250%, subfraction 3 by 250%, and subfraction 4 by 240%. Although oAp_4A increased [^{3}H]ryanodine binding from 10.1 ± 2.6 to 18.4 ± 3.8 (180%) in subfraction 5 and from 10.8 ± 3.1 to 14.1 ± 4.4 in subfraction 6 (130%), these increases were not statistically significant. Compared with

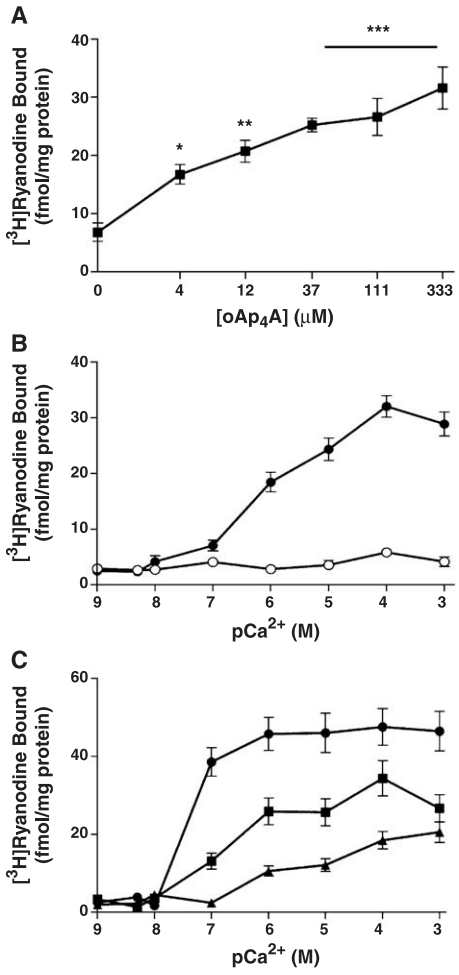

Fig. 1. (A) Effects of oAp_4A on [^{3}H]ryanodine binding in microsomal fractions of rat brain. Data shown are the mean ± S.E.M. from five different tissue preparations. *, **, ***—significantly greater ($p < 0.05$, 0.01, 0.001) than control value. (B) Effects of free Ca^{2+} on the enhancement of [^{3}H]ryanodine binding by oAp_4A. Shown are means ± S.E.M from four different microsomal preparations assayed in the absence (open circles) or presence (filled circles) of 111 μM oAp_4A. (C) Effects of 20 mM caffeine (circles), 1.2 mM Mg^{2+} (squares), and 5.0 mM Mg^{2+} (triangles) on [^{3}H]ryanodine binding in the presence of 111 μM oAp_4A. Shown are the mean ± S.E.M. from four different microsomal preparations.

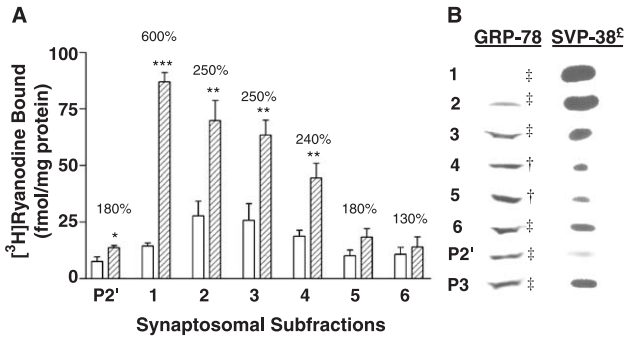

Fig. 2. (A) Effects of 111 μM oAp_4A (hatched bars) on [^{3}H]ryanodine binding in synaptosomal subfractions (1 to 6) and total synaptosome fraction (P2′) isolated from rat brain. Data shown are the mean ± S.E.M. from three to five different tissue preparations. Shown are percent activation over control (clear bars) values. *, **, ***—significantly greater ($p < 0.05$, 0.01, 0.001, respectively) than corresponding control. (B) Immunoblot analysis of synaptosomal subfractions shown in A. Blots were probed using microsome (GRP-78) or synaptic vesicle (synaptophysin, SVP-38) markers. Each band represents either 20 (‡), 10 (†), or 1.5 (£) μg of total protein loaded. Although no signal for GRP-78 was detected in this tissue preparation, analysis of other preparations carried out in the same fashion revealed, albeit at very low levels, GRP-78 in subfraction 1. Shown are results representative of analyses carried out on three separate tissue preparations.

P2′, [^{3}H]ryanodine binding in the presence of oAp_4A was significantly ($p < 0.01$) higher in subfractions 1, 2, 3, and 4 but not in subfractions 5 and 6.

4. Discussion

In previous studies, we showed that Ap_nAs, and more potently, oAp_nAs, increase [^{3}H]ryanodine binding to ryanodine receptors in total membrane preparations of rat brain (Holden et al., 1996). Here we confirmed and extend our previous results showing that [^{3}H]ryanodine-binding activity in synaptosomal subfractions was highest in lightest subfractions and declined in heavier ones (Padua et al., 1996). Immunoblot analyses of synaptosomal subfractions showing elevated levels of [^{3}H]ryanodine binding in subfractions with high levels of synaptic vesicle-associated protein and very low or absent levels of GRP-78 suggested an association between ryanodine receptors and synaptic vesicles.

Adenine nucleotides are known to increase [^{3}H]ryanodine binding to ryanodine receptors (Holden et al., 1996; Padua et al., 1994; Pessah et al., 1987), and in this group of compounds, Ap_nAs have the most potent effects (Holden et al., 1996). Here, using oAp_4A, we showed that Ap_nAs had the most potent effect on ryanodine receptors in synaptosomal subfractions containing the highest levels of synaptophysin. In increasing [^{3}H]ryanodine binding, our studies using microsomal fractions suggested that oAp_4A acted by increasing the population of RyRs in a Ca^{2+}-activated state given that ryanodine binds preferentially to the open state of the ryanodine receptor. Further, our finding that the maximum level of [^{3}H]ryanodine binding occurred at the same Ca^{2+} concentration (100 μM) in the absence or presence of oAp_4A suggested that these compounds were not affecting the Ca^{2+} activation threshold of ryanodine receptors as do the other modulators such as Mg^{2+} and caffeine (Henzi and MacDermott, 1992). These findings are consistent with others showing that adenine nucleotides modulate ryanodine receptors by sustaining the Ca^{2+}-activated form of the receptors (Ashley, 1989; Laver et al., 2001).

Previous use of periodate-oxidized nucleotides as affinity labels for receptors (Low et al., 1992), as enzyme inhibitors in place of the corresponding non-oxidized endogenous substrates (Borchardt et al., 1978; Easterbrook-Smith et al., 1976; Magos et al., 1987), and as receptor agonists (Dalpiaz et al., 1995) has demonstrated that oxidation of the ribose moiety increases the interaction of the endogenous nucleotide molecule for its protein target without compromising the specificity of its interaction with that target. Our results using oAp_4A show that the function of synaptic ryanodine receptors can be significantly altered by adenine nucleotides, specifically Ap_nAs. Under conditions where Ap_nA levels increase, such as during periods of cell proliferation (Rapaport and Zamecnik, 1976; Vartanian et al., 1997; Weinmann-Dorsch et al., 1984) or under conditions of cell stress (Baker and Jacobson, 1986; Jovanovic et al., 1998; Lee et al., 1983) and neuronal activity, $[Ca^{2+}]_i$ signaling through ryanodine sensitive pools may be increased.

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