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Failure of adenosine analogues to affect N-type voltage sensitive Ca^{2+} channels in chicken brain synaptosomes

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Adenosine (or ATP) is released, along with neurotransmitters, from nerves in the central nervous system [1]. These nucleotides (and their analogues) inhibit further neurotransmitter release by negative feedback on presynaptic adenosine A_1 receptors [2, 3]. Several mechanisms have been proposed to explain this activity. One hypothesis suggests that adenosine analogues interfere with Ca^{2+} influx [4–6] or promote Ca^{2+} sequestration in the presynaptic terminal [7]. However, reports of the effects of these compounds on Ca^{2+} influx have been inconsistent [4–6, 8–10], while the direct measurement of the effect of these compounds on intraterminal free Ca^{2+} concentrations ($[Ca^{2+}]_{int}$) has not, to our knowledge, been performed in neural tissue. Another possibility has been suggested [11, 12]: that adenosine analogues may preferentially inhibit Ca^{2+} flux through N-type voltage sensitive Ca^{2+} channels (VSCCs*), an alternative that also has not been directly explored.

We first studied the effects of the A_1 agonists 2-chloroadenosine (2-CADO) and N^6-cyclohexyladenosine (CHA) on Ca^{2+} movements and ^{125}I-labeled ω-conotoxin (^{125}I-ω-CgTx) binding to the N channel in rat brain preparations. These analogues were found to have no effect on any aspects of Ca^{2+} influx studied, confirming the results of Barr *et al.* [8] and Garritson *et al.* [9]. Further, extending the previous investigations, these analogues had no effect on the rise in $[Ca^{2+}]_{int}$ evoked by K^+ or on ω-CgTx binding in rat brain preparations. Since the N channel inhibitor ω-CgTx tends to have relatively minor effects on Ca^{2+} influx in rat brain [13], we deemed it necessary to repeat the experiments in chicken brain for the following reasons: chicken brain is rich in adenosine A_1 receptors [14], and, unlike the situation in rat brain, inhibition of the N-type Ca^{2+} channel in chicken brain with ω-CgTx blocks virtually all of the parameters of Ca^{2+} mobility investigated in this species [15–17]. The present results deal only with the effects of A_1 agonists in chicken brain preparations, but are qualitatively similar to results we obtained in rat brain.

The $^{45}Ca^{2+}$ influx studies were carried out essentially by the method of Blaustein [18]. The effect of ω-conotoxin, 2-CADO or CHA on Ca^{2+} influx was studied under resting (5 mM K^+) and depolarizing conditions (40 mM K^+), and the K^+-stimulated component (40 mM minus 5 mM) was calculated.

$[Ca^{2+}]_{int}$ was estimated in chicken brain preparations essentially as described by Komulainen and Bondy [19]. Briefly, the synaptosomal F_4 pellet, isolated using the Percoll gradient technique of Robinson and Lovenberg [20],

* Abbreviations: VSCCs, voltage sensitive Ca^{2+} channels; CHA, N^6-cyclohexyladenosine; 2-CADO, 2-chloroadenosine; ω-CgTx, ω-conotoxin GVIA; and EGTA, ethyleneglycol-bis-(β-aminomethyl ether)tetra-acetate.

was resuspended in 1 mL of Krebs solution containing a 2 μM concentration of the Ca^{2+} sensitive dye acetoxymethyl-FURA-2 (FURA-2-AM) which was incubated for 20 min at 37°. This solution was then diluted 10-fold with Krebs solution and incubated for a further 20 min. The synaptosomes were collected by centrifugation for 5 min at 2000 *g* and resuspended in Na^+-Krebs or choline-Krebs buffer at a concentration of 0.7 to 1.0 mg/mL. Aliquots (200 μL) of this solution were centrifuged for 10 sec in an Eppendorf micro-centrifuge before final dilution with 2.4 mL of the appropriate Krebs solution and placement in the spectrofluorometer. Drug incubations were performed in 200 μL prior to the final dilution, and the appropriate concentration was maintained in the cuvette (except in the case of ω-CgTx which was not replaced in the final 2.4 mL).

F_4 synaptosomes were suspended in 50 mM Tris–HCl, pH 7.4, and homogenized for 30 sec at setting 6 using a Polytron homogenizer. The synaptic plasma membranes (SPM) were collected at 30,000 *g* by centrifugation, washed twice by resuspension in Tris–HCl, and finally resuspended in Tris–HCl for use in the binding assay.

^{125}I-ω-CgTx (sp. act. 2200 Ci/mmol, New England Nuclear, Mississauga, Ontario) was obtained as a lyophilized powder and diluted into distilled water. Labelled ω-CgTx (20 pM) was incubated with 2 μg SPM and various concentrations of the adenosine analogues (total volume 500 μL) for 60 min at 37°. The reaction was stopped using ice-cold 50 mM Tris–HCl containing 0.02% bovine serum albumin (BSA). The membranes were collected on Gelman GF-C filter paper using a Brandel Cell Harvester (Brandel, Gaithersburg, MD), washed twice with the quench buffer and counted using a Beckman Gamma Counter (model 4000). Non-specific binding was determined in the presence of 10 nM ω-CgTx. Protein concentration was determined by the method of Bradford [21] using reagents purchased from Bio-Rad (Richmond, CA).

Ca^{2+} influx, measured at various time intervals, with or without drug treatments, was compared by two-way analysis of variance. Similarly, intracellular free Ca^{2+} concentrations in control and treated synaptosomes were compared by analysis of variance followed by Dunnet's multiple range test. A significant difference was assumed if $P \leq 0.05$.

Chicken brain synaptosomes were incubated alone or in the presence of 2-CADO or CHA (10 μM) and in the presence of adenosine deaminase in either choline or Na^+ buffer (results using Na^+ buffer not shown). After a 15-min preincubation, Ca^{2+} influx was measured for from 1 to 20 sec following depolarization with 40 mM K^+. Neither of the adenosine analogues reduced $^{45}Ca^{2+}$ influx in either buffer significantly, in marked contrast to the large decrease (between 65 and 90%) resulting from a 15-min preincubation of synaptosomes with ω-CgTx (0.1 μM) (Fig. 1).

The effects of depolarizing FURA-2 loaded synaptosomes in the presence of CHA or 2-CADO (10 μM) or ω-CgTx (0.1 μM) are shown in Fig. 2. As was the case when $^{45}Ca^{2+}$ influx was measured, the adenosine analogues failed to decrease the K^+-induced rise in $[Ca^{2+}]_{int}$, whereas ω-CgTx inhibited the rise by approximately 50% at a concentration 2 orders of magnitude less than the adenosine analogues.

Although the adenosine analogues failed to inhibit ω-CgTx sensitive Ca^{2+} influx or $[Ca^{2+}]_{int}$ levels, we nonetheless examined the possibility that 2-CADO or CHA might modify ω-CgTx binding to the N channel (Fig. 3). ω-CgTx binding was measured in synaptosomal membranes and compared with binding measured in the presence of various concentrations of CHA or 2-CADO (1–100 μM) (Fig. 3). The specific binding of ^{125}I-ω-CgTx was not inhibited significantly by the adenosine analogues at any concentration up to 100 μM. In fact, there was a tendency for the adenosine analogues to promote binding which

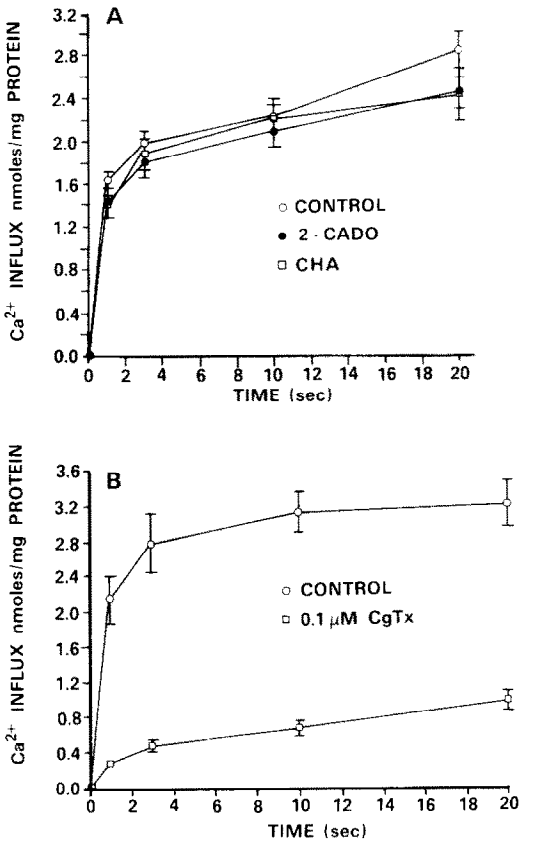

Fig. 1. Effects of adenosine analogues and ω-CgTx on Ca^{2+} influx in synaptosomes. (A) Synaptosomes prepared from chicken brain were incubated in choline resting buffer (RB), or preincubated in the same buffer containing 10 μM 2-CADO or CHA. Aliquots were removed and were injected into depolarizing buffer (40 mM K^+) containing $^{45}Ca^{2+}$, and influx was allowed to continue for various times followed by termination using quench buffer (RB with 4 mM EGTA) and rapid filtration. (B) The same experiment was carried out following preincubation of the synaptosomes with ω-CgTx (0.1 μM). Each point is the mean Ca^{2+} accumulation ± SE from four experiments performed in triplicate.

achieved statistical significance ($P \leq 0.05$) at the low dose of 2-CADO.

N-type Ca^{2+} channels have been characterized recently in neuronal tissue and largely, if not solely, regulate the influx of Ca^{2+} and the subsequent rise in $[Ca^{2+}]_{int}$ [22]. The criteria which define the presence of N channels in the preparations utilized in the present work have been described previously [15–17] and, therefore, the VSCC studied here can safely be referred to as N-type VSCCs.

Information concerning the effect of adenosine or its analogues on Ca^{2+} influx is inconsistent. 2-CADO has been reported to be a potent inhibitor of $^{45}Ca^{2+}$ influx, with an IC_{50} of 0.02 μM [4, 6], a weak inhibitor with an IC_{50} of 50 μM [5], or not an inhibitor at all [8, 9].

The present experiments, designed to investigate potential effects of adenosine derivatives on Ca^{2+} metabolism, were carried out at time periods relevant for measuring both fast phase (3 sec; thought to specifically represent Ca^{2+} accumulation through membrane channels) and slow phase (>3 sec; representing influx which may be contaminated by mechanisms such as Na^+/Ca^{2+} exchange)

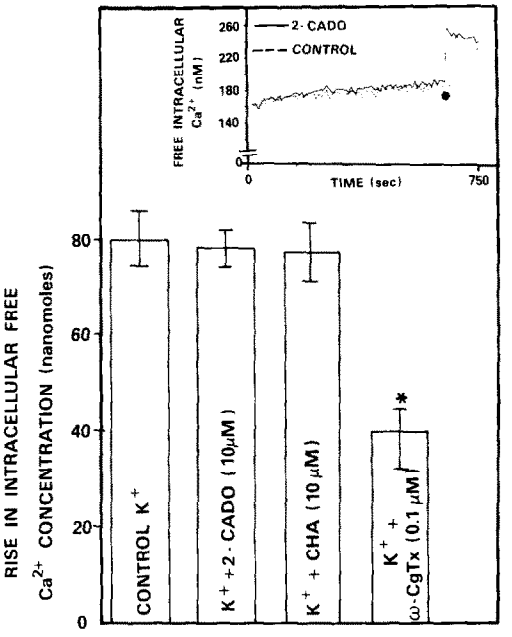

Fig. 2. Effects of 2-CADO, CHA and ω-CgTx on $[Ca^{2+}]_{int}$ estimated with the Ca^{2+} sensitive dye, FURA-2. FURA-2 loaded synaptosomes from chicken brain were depolarized with 21 mM K^+, and the rise in $[Ca^{2+}]_{int}$ was measured. In other experiments, the synaptosomes were preincubated with 10 μM 2-CADO or CHA or 0.1 μM ω-CgTx prior to the depolarizing stimulus. Each bar represents the mean ± SE from three preparations. Key: (*) significantly different from control ($P \leq 0.05$). The inset shows the effect on $[Ca^{2+}]_{int}$ following K^+ (21 mM) stimulation in control and 2-CADO-treated synaptosomes.

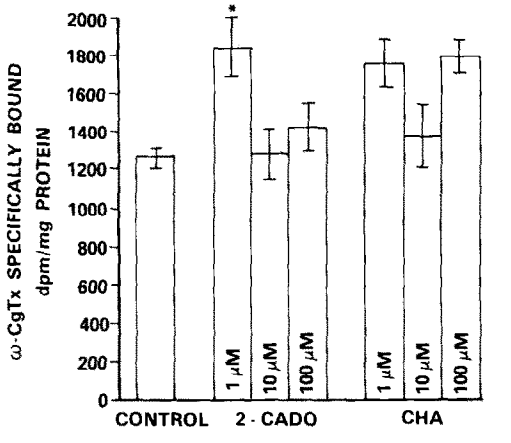

Fig. 3. Specific binding of ^{125}I-ω-CgTx to chicken synaptic plasma membranes measured in the presence of various concentrations of 2-CADO or CHA. The bars represent mean specific ω-CgTx binding ± SE from three preparations in duplicate. Key: (*) 1 μM 2-CADO value was significantly different from control ($P \leq 0.05$).

[23]. In addition, adenosine deaminase was added to the buffers to minimize the contribution of endogenous nucleosides, and Na^+ was replaced by choline in the Krebs buffer to reduce the contribution of non-VSCC-mediated Ca^{2+} accumulation mechanisms. Under these experimental conditions, no diminution of Ca^{2+} influx could be determined in the presence of high concentrations of either 2-CADO or CHA. No reduction of influx was evident at any time period of depolarization examined in either choline buffer or Na^+ buffer (latter results not shown). Identical studies were performed using low concentrations of ω-CgTx, orders of magnitude lower than the adenosine derivatives, as a positive control and for comparative purposes. ω-CgTx produced substantial and significant inhibition of Ca^{2+} influx. Qualitatively identical results were obtained with the adenosine analogues (i.e. no effect) using rat brain synaptosomes (results not shown).

Although measurements were not carried out, the suggestion has been made that rather than affecting Ca^{2+} influx, adenosine or its derivatives may affect $[Ca^{2+}]_{int}$ levels [7]. In the present study, neither analogue had any discernible effect on the concentration of $[Ca^{2+}]_{int}$. On the other hand ω-CgTx effectively inhibited K^+-stimulated increases in $[Ca^{2+}]_{int}$ at concentrations as low as 0.03 μM (results not shown, [16]) which were between 2 and 3 orders of magnitude less than the concentrations of the adenosine analogues found to have no effect. The failure of these adenosine analogues to affect intracellular concentrations of Ca^{2+} also suggests that, under our conditions, they fail to affect intracellular mechanisms of binding or elimination of Ca^{2+} in neuronal tissue. Furthermore, the binding of ^{125}I-ω-CgTx to synaptic plasma membranes was not inhibited by the adenosine analogues, as would have been expected if these compounds had affected the N-type, ω-CgTx sensitive, Ca^{2+} channel.

The experiments presented here address suggestions raised by others [24, 25] that adenosine A_1 agonists may affect the N-type Ca^{2+} channel. It is evident from the above discussion and recent publications that considerable controversy still exists concerning the ability of A_1 receptor activation to inhibit Ca^{2+} influx. It may be, as suggested by Fredholm *et al.* [24], that adenosine analogues affect Ca^{2+} influx depending on such factors as tissue source, neuronal type, or depolarization stimulus. Without comment on the relative contribution of these factors, we conclude that under the conditions described here, no reduction of Ca^{2+} influx through VSCCs of the N type could be demonstrated. These results are consistent with several studies that have suggested a component in the activity of adenosine analogues which is unrelated to Ca^{2+} influx (see Silinsky [26]), and appear similar to results which showed a relatively small inhibition by 2-CADO (approximately 20%) recently demonstrated on the N channel in electrophysiological experiments [27].

In summary, a controversial hypothesis suggests that adenosine and its derivatives inhibit Ca^{2+} influx and the associated rise in intracellular Ca^{2+} concentrations in neural tissue. We have re-examined this proposal utilizing a neuronal preparation with well-defined functional N-type Ca^{2+} channels. The results offer no support for the suggestion that adenosine or its analogues inhibit N-type voltage sensitive Ca^{2+} channels.

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Possible chloroquine-induced modification of N-acetylation of isoniazid and sulphadimidine in the rat

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Chloroquine, a 4-aminoquinoline derivative widely used in the treatment of malaria [1] affects a wide range of biochemical processes in the living cell [2–4]. For instance, it is known to alter the permeability of the lysosomes resulting in increased levels of hydrolytic enzymes [5]. It also interferes with protein synthesis [6]. We have previously reported the chloroquine-induced alterations in rat hepatic microsomal components resulting in decreased activity of some of the drug oxidizing enzymes which we suggested could arise from changes in the endoplasmic reticulum membrane fluidity [7].

Following an interest in the consequences of the combined administration of isoniazid (INH) or sulphadimidine (SDM) with chloroquine to tuberculosis patients during