*Biochemical Pharmacology*, Vol. 34, No. 5, pp. 713–715, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00


# Synaptosomal calcium uptake unaltered by adenosine and 2-chloroadenosine

(*Received* 2 *May* 1984; *accepted* 20 *August* 1984)

Purinergic compounds can decrease neural activity peripherally and centrally [1–4]. The spontaneous firing rates of cerebellar Purkinje neurons fall precipitously after adenosine or its analogs are applied locally [5]. The amplitude of hippocampal excitatory postsynaptic potential (EPSP) responses is depressed by adenosine and its analogs [5, 6]. The depression of EPSP amplitudes of pyramidal neurons of the hippocampus is unaccompanied by changes of resting membrane potential or resistance following perfusion with 5 $\mu$M adenosine, implying presynaptic effects, but treatment with 10 $\mu$M adenosine produces a hyperpolarization with a reduction of input resistance [6]. Hyperpolarization without a change of resistance has been reported for cerebral cortical neurons after iontophoresis of adenosine 5′-monophosphate [7].

Neurotransmitter release is decreased by purinergic compounds. Adenosine (0.01 mM) reduces the depolarization-induced release of dopamine, acetylcholine and 5-hydroxytryptamine from striatal slices [8]. Adenosine triphosphate (0.1 to 0.5 mM) inhibits the release of acetylcholine, as shown by decreased synaptic responses of sympathetic ganglion cells, but adenosine (0.4 to 0.75 mM) is without effect on these responses [9]. Adenosine is suggested to reduce neurotransmitter release by blockade of calcium channels [2]. The suggestion of calcium involvement in the reduction of transmitter release is based upon reports of adenosine-induced inhibition of calcium uptake by synaptosomes [10, 11]. To further evaluate this possibility of presynaptic calcium mechanisms, we examined the effects of adenosine and 2-chloroadenosine on fast- and slow-phase voltage-dependent calcium entry into synaptosomes.

## *Materials and methods*

Synaptosomes were isolated from the whole brain of male Sprague-Dawley rats (200–300 g), similar to previous description [12], and resuspended in incubation medium (NaCl, 136 mM; KCl, 5 mM; $CaCl_2 \cdot 2H_2O$, 1.2 mM; $MgCl_2$, 1.3 mM; glucose, 10 mM; Tris base, 20 mM; pH adjusted to 7.65 with 1 M maleic acid) to provide a protein concentration of approximately 1–2 mg/ml. After resuspension, 0.48-ml aliquots of the synaptosomal preparations were pipetted into sample tubes and incubated for approximately 14 min in a Dubnoff shaker at 30° in the presence or absence of adenosine or 2-chloroadenosine (Sigma Chemical Co., St. Louis, MO). Adenosine or 2-chloroadenosine was added in a 0.02 ml volume to make a final incubation volume of 0.5 ml. The final concentration of adenosine was 0.01 mM and that of 2-chloroadenosine was either 0.01 or 0.1 mM. Adenosine concentration in brain ranges from approximately 0.002 to 0.015 mM [13]. For control samples, 0.02 ml of incubation medium was added. For time–response experiments, 0.5-ml aliquots of depolarizing or nondepolarizing solutions containing $^{45}Ca^{2+}$ (3 $\mu$Ci of $^{45}Ca^{2+}$/$\mu$mole of $^{40}Ca^{2+}$) were added for times ranging from 1 to 15 sec. The composition of the depolarizing solution was the same as the incubation medium except that NaCl was isosmotically replaced by KCl to provide a final KCl concentration of 65 mM. The nondepolarizing solution had the same composition as the incubation medium except for the presence of 3 $\mu$Ci of $^{45}Ca^{2+}$/$\mu$mole of $^{40}Ca^{2+}$. Depolarizing and nondepolarizing solutions which were added to purine-exposed synaptosomes also contained the same purine concentration to maintain the designated molar relationships. At the appropriate times (1, 3, 5 or 15 sec), uptake of $^{45}Ca^{2+}$ was terminated by the prompt addition of 5 ml of ice-cold ethylene glycol bis-($\beta$-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) "stopping solution" (NaCl, 136 mM; KCl, 5 mM; $MgCl_2$, 1.3 mM; EGTA, 3 mM; glucose, 10 mM; Tris base, 20 mM; pH adjusted to 7.65 with 1 M maleic acid). Each sample was immediately filtered through a presoaked 25-mm Whatman GF-B filter using a Millipore sampling manifold. Each filter was washed with two 5-ml aliquots of ice-cold incubation medium, placed in a scintillation vial containing 10 ml of Ready-Solv EP, and counted in a Beckman LS-1800 liquid scintillation counter. Experiments examining 1-, 3- and 5-sec uptake periods were timed using a Franz electronic metronome. To calculate the net uptake of $^{45}Ca^{2+}$ into synaptosomes, the uptake in the absence of depolarization (5 mM KCl) was subtracted from the uptake in the presence of depolarization (65 mM KCl). This value will be referred to as $\Delta_k$ (potassium-induced change) and represents net KCl-induced $^{45}Ca^{2+}$ uptake [14]. Each control and purine experiment was performed using matched synaptosomal preparations obtained from the same batch. Synaptosomal protein concentration was determined by the method of Oyama and Eagle [15].

## *Results and discussion*

The values of our measurements of calcium uptake for the various times are shown in Table 1 and Fig. 1. The synaptosomes in nondepolarizing solutions treated with adenosine (0.01 mM) or 2-chloradenosine (0.01 or 0.1 mM) showed no significant differences in calcium uptake from the matched control values at each of the measured times (Table 1). These results are consistent with the lack of effect of adenosine or 2-chloroadenosine on norepinephrine release by PC12 cells in the absence of depolarization with potassium [16]. Figure 1 shows no significant differences between net values of calcium uptake by depolarized synaptosomes for control and purine-treated groups at the various times, except for one comparison. Adenosine (0.01 mM) appears to inhibit calcium uptake by depolarized synaptosomes only at 5 sec of uptake. No significant effect on calcium uptake was observed at 5 sec or any other uptake time using 2-chloroadenosine, a more potent analog [3, 17] than adenosine. The physiological significance of this one adenosine inhibition becomes more suspect in considering the failure of adenosine to inhibit calcium uptake at the other measured times before or after 5 sec.

Fast-phase calcium uptake is known to occur within the first 1–3 sec following depolarization [18, 19] and appears to be related to initial calcium influx and phasic neurotransmitter release. A slow-phase calcium uptake process follows at 5 sec or greater [18–20]. During the fast phase, we saw no significant differences between net values of depolarization-induced calcium uptake by control and purine-exposed synaptosomes (see Fig. 1). Our longest time of measurement was 15 sec which is well within the interval of slow-phase processes for calcium uptake [18–20]. Measurement of the slow-phase processes of synaptosomal calcium uptake in the presence or absence of purinergic drugs is reported by other groups [10, 11] at uptake times between 15 and 120 sec. At 30 sec 0.1 mM adenosine concentration is reported to decrease calcium uptake [10]. At a 20-sec measurement time, Wu *et al.* [11] reported an inhibition of calcium uptake by depolarized synaptosomes treated with adenosine or 2-chloroadenosine. These results from 20- and 30-sec measurements conflict with our 15-sec

Table 1. Effects of adenosine or 2-chloroadenosine treatment on non-stimulated $Ca^{2+}$ uptake by whole brain synaptosomes*

| Time (sec) | No drug | Ado (0.01 mM) | 2-Cl-Ado (0.1 mM) | 2-Cl-Ado (0.01 mM) |
|---|---|---|---|---|
| | $Ca^{2+}$ uptake (nmoles $Ca^{2+}$/mg protein) Nondepolarized uptake | | | |
| 1 | 0.89 ± 0.24 | 0.66 ± 0.25 | 0.72 ± 0.29 | 0.86 ± 0.37 |
| 3 | 1.22 ± 0.17 | 1.25 ± 0.29 | 1.06 ± 0.31 | 1.38 ± 0.49 |
| 5 | 1.65 ± 0.19 | 1.75 ± 0.35† | 1.64 ± 0.37 | 2.01 ± 0.50 |
| 15 | 2.64 ± 0.34 | 2.35 ± 0.41 | 2.49 ± 0.48 | 2.63 ± 0.40 |

* Values are means ± S.E.M. of four experiments each performed with duplicate samples. Nondepolarized data represent $^{45}Ca^{2+}$ accumulation by resting synaptosomes (5 mM KCl). Abbreviations: Ado, adenosine; and 2-Cl-Ado, 2-chloroadenosine.

† Significant difference between the purine-altered and the control uptake value as shown by Student's paired *t*-test (two-tailed) at the 0.05 level.

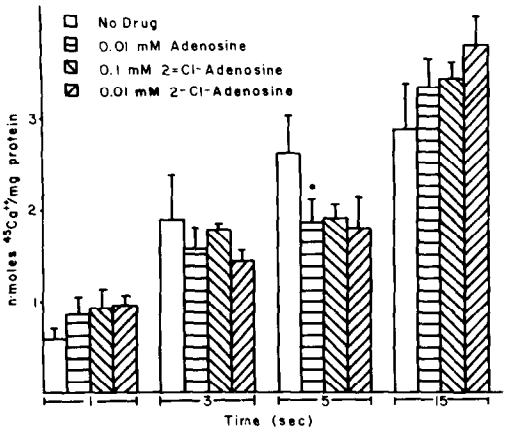


Fig. 1. Effects of adenosine and 2-chloroadenosine treatment on net KCl-stimulated $Ca^{2+}$ uptake by whole brain synaptosomes. Values are means ± S.E.M. of four experiments, each performed with duplicate samples. Net uptake ($\Delta_K$) values were calculated as the difference between appropriate KCl-depolarized and nondepolarized data and represent the net synaptosomal $Ca^{2+}$ uptake in response to depolarization. The asterisk refers to a significant difference between the purine-altered and control uptake value as shown by Student's paired *t*-test (two-tailed) at the $P < 0.05$ level.

uptake results. The slow-phase process predominates in the 15- to 30-sec time intervals of all these measurements of calcium uptake by depolarized synaptosomes [18–20]. Examination of the 15-sec measurement for 0.1 and 0.2 mM adenosine data from Ribeiro *et al.* [10] shows no apparent adenosine-induced decrease in calcium uptake by synaptosomes, in agreement with our results. At 1.0 or 2.0 mM adenosine or at uptake measurement times greater than 15 sec, Ribeiro *et al.* show decreased calcium uptake. The causes of the observed differences between our group and Wu *et al.* [11] or Ribeiro *et al.* [10] are uncertain. Glial cells are commonly present in the suspensions but glial cell uptake of calcium in high potassium solutions is negligible compared to that for depolarized synaptosomes [21]. Glial cell contamination is, therefore, not a source of the reported differences of purinergic effects on calcium uptake by depolarized synaptosomes. Procedural differences between our measurements and those of previous reports [10, 11] are small and do not explain the conflicting data.

To explain the purinergic inhibitory effects on electrical activity or transmitter release, various mechanisms are proposed [22]. These include reduction of depolarization-induced calcium influx in presynaptic terminals [10, 11], hyperpolarization of the presynaptic membrane, or interference with excitation–secretion coupling perhaps through mechanisms involving cAMP [23]. The hyperpolarizations and decreased transmitter release may be related phenomena. Changes in membrane potential are reported to control transmitter release [24, 25]. Our results suggest that adenosine does not alter calcium uptake by isolated depolarized nerve endings. This finding is important in view of the commentary in a recent review that adenosine neuromodulation in the central nervous system may be linked with calcium entry blockade [5].

*Division of Pharmacology*
*College of Pharmacy*
*The University of Texas at Austin*
*Austin, TX 78712, U.S.A.*

EDWARD BARR*
LAURA C. DANIELL
STEVEN W. LESLIE

* Address correspondence to: Edward Barr, Clayton Foundation Biochemical Institute, University of Texas at Austin, Austin, TX 78712.

REFERENCES

1. J. W. Daly, R. F. Bruns and S. H. Snyder, *Life Sci.* **28**, 2083 (1981).
2. J. W. Phillis and P. H. Wu, *Prog. Neurobiol.* **16**, 187 (1981).
3. T. W. Stone, *Neuroscience* **6**, 523 (1981).
4. C. Su, *A. Rev. Pharmac. Toxic.* **23**, 397 (1983).
5. T. V. Dunwiddie, A. S. Basile and M. R. Palmer, *Life Sci.* **34**, 37 (1984).
6. G. R. Siggins and P. Schubert, *Neurosci. Lett.* **23**, 55 (1981).
7. J. W. Phillis, J. P. Edstrom, G. K. Kostopoulos and J. P. Kirkpatrick, *Can. J. Physiol. Pharmac.* **57**, 1289 (1979).

8. H. H. Harms, G. Wardeh and A. H. Mulder, *Neuropharmacology* **18**, 577 (1979).
9. E. M. Silinsky and B. L. Ginsborg, *Nature, Lond.* **305**, 327 (1983).
10. J. A. Ribeiro, A. M. Sá-Almeida and J. M. Namorado, *Biochem Pharmac.* **28**, 1297 (1979).
11. P. H. Wu, J. W. Phillis and D. L. Thierry, *J. Neurochem.* **39**, 700 (1982).
12. S. V. Elrod and S. W. Leslie, *J. Pharmac. exp. Ther.* **212**, 131 (1980).
13. H. R. Winn, R. Rubio and R. M. Berne, *Am. J. Physiol.* **241**, H235 (1981).
14. S. W. Leslie, M. B. Friedman, R. E. Wilcox and S. V. Elrod, *Brain Res.* **185**, 409 (1980).
15. V. I. Oyama and H. Eagle, *Proc. Soc. exp. Biol. Med.* **91**, 305 (1956).
16. C. S. Rabe and R. McGee, *J. Neurochem.* **41**, 1623 (1983).
17. K. G. Thampy and E. M. Barnes, *J. Neurochem.* **40**, 874 (1983).
18. S. W. Leslie, E. Barr and L. J. Chandler, *J. Neurochem.* **41**, 1602 (1983).
19. J. Gripenberg, E. Heinonen and S. E. Jansson, *Br. J. Pharmac.* **71**, 265 (1980).
20. D. A. Nachshen and M. P. Blaustein, *J. gen. Physiol.* **76**, 709 (1980).
21. E. M. Barnes and P. Mandel, *J. Neurochem.* **36**, 82 (1981).
22. M. L. Michaelis, E. K. Michaelis and S. L. Myers, *Life Sci.* **24**, 2083 (1979).
23. D. Van Calker, M. Muller and B. Hamprecht, *J. Neurochem.* **33**, 999 (1979).
24. J. Dudel, *Neurosci. Lett.* **41**, 133 (1983).
25. J. Dudel, I. Parnas and H. Parnas, *Pflügers Arch.* **399**, 1 (1983).