Biochemical Pharmacology, Vol. 28, pp. 1297–1300.

0006-2952/79/0415-1297 $05.00/0

ADENOSINE AND ADENOSINE TRIPHOSPHATE DECREASE ^{45}Ca UPTAKE BY SYNAPTOSOMES STIMULATED BY POTASSIUM

J. A. RIBEIRO, A. M. SÁ-ALMEIDA and J. M. NAMORADO
Centro de Biologia, Instituto Gulbenkian de Ciência, Oeiras, Portugal

(*Received* 25 *September* 1978; *accepted* 31 *October* 1978)

Abstract—The action of adenosine and adenosine triphosphate on the uptake of ^{45}Ca has been studied on the synaptosome-rich preparation from the rat cerebral cortex. The preparation was depolarized by high potassium. Adenosine (0.1–2 mM) and adenosine triphosphate (0.2 mM) decreased the uptake of ^{45}Ca. Adenosine triphosphate (1 mM) did not change significantly the uptake of ^{45}Ca and adenosine triphosphate (2 mM) even increased it. These results indicate that the inhibitory effects of these substances on neurotransmission result from its inhibitory effects on the entry of calcium into the nerve endings.

A physiological role for adenosine and ATP as possible modulators of the transmitter release has been frequently put forward on the basis that a mechanism involving the release of ATP and related substances (ADP, AMP and adenosine) in different nervous systems, either centrally or peripherally, autonomic or motor, may control the output of acetylcholine (Ach) or noradrenaline (NA), modulating by a negative feedback mechanism in some part the release of these neurotransmitters (see ref. 1). Adenosine and ATP in both rat phrenic nerve-diaphragm and frog-sartorius preparations reduced maximally to about half the control values of the evoked release of the transmitter, as measured by the ratio of the mean amplitude of evoked end-plate potentials (e.p.p.'s) to the mean amplitude of miniature e.p.p.'s, without changing significantly the mean amplitude of miniature e.p.p.'s [2, 3]. Similar qualitative and quantitative results were obtained using those substances which decrease the amplitude of evoked post-synaptic potentials in the olfactory cortex slices [4, 5]. On the other hand, it is well established that the evoked release of the transmitter depends on the entry of calcium ions into the presynaptic nerve terminals during depolarization at both peripheral [6] and central [7] nervous systems. Thus an explanation for the action of those substances should consider the "calcium hypothesis", implicating therefore that a modulation of the transmitter release by adenosine or ATP depends on a modulation of calcium entry into the nerve endings. However, such a hypothesis cannot be tested using the rat motor nerve endings as a preparation, since it cannot be isolated from the muscle. We have therefore examined the effects of adenosine and ATP on the uptake of ^{45}Ca by synaptosomes, since this preparation retains many of the functional properties of intact neurones [7]. The results reported here show that adenosine and ATP decrease the ^{45}Ca uptake by synaptosomes stimulated by potassium.

MATERIALS AND METHODS

Solutions. The composition and nomenclature of representative solutions are shown in Table 1. Intermediate concentrations of alkali metal ions were obtained by mixing the appropriate solutions (see legend of Fig. 1). The EGTA stopping solution (in H_2O) was titrated to pH 7.4 (at 25°) with other EGTA solution (in Tris base) with the same molarity and contained (mM): NaCl 120; KCl 5 and EGTA 30. The sucrose solutions were corrected to pH 7.4 with 0.1 M NaOH. All solutions were prepared with de-ionized glass-distilled water.

Table 1. Composition of representative solutions*

Solution	NaCl (mM)	KCl (mM)	$CaCl_2$ (mM)	Choline Cl (mM)
Na + 5K	132	5	1.2	0
Ca-free Na + 5K	132	5	0	0
Choline + 5K	0	5	1.2	132
137 mM-K saline	0	137	1.2	0

* In addition to the components listed, all solutions contained (mM): $MgCl_2$ 1.3; NaH_2PO_4 1.2; glucose 10 and Tris base 20. The solutions were buffered to pH 7.4 (at 25°) by titration with maleic acid.

Chemicals. Tris (hydroxymethyl) aminomethane; ethylene glycol-bis [β-aminoethyl ether] *N,N'*-tetracetic acid (EGTA); adenosine and adenosine 5'-triphosphate (ATP) were purchased from Sigma. Triton X-100 (scintillation grade) and toluene (scintillation grade) were from BDH. Other reagents were analytical grade from BDH. 2.5 Diphenoxyloxazole (PPO) was purchased from NEN Chemicals, and 1,2-bis-2-[5-phenoxyloxazole]benzene (POPOP) was a Packard International product. $^{45}CaCl_2$ was purchased from Radiochemical Centre, Amersham.

Preparation of synaptosomes. Synaptosomes were prepared from whole rat brain homogenates (4 rats of either sex, ~ 40 days old, per experiment), by the differential centrifugation and sucrose density gradient centrifugation procedures described by Gray and Whittaker [8], slightly modified to reduce microsomal contamination in the mithocondrial pellet (P_2) [9]. Homogenization of brains was made in ice cold sucrose 0.32 M (10% w/v), in a glass and Perspex homogenizer (rotating at 840 rev/min) with a difference in diameter of 0.025 cm between pestle and mortar (Braun Melsungen). The homogenate was first centrifuged at 1000 *g* for 10 min at 4°. The supernatant (S_1) was centrifuged for only 25 min at 10.000 *g* and 4°. The resulting pellet (P_2) was re-suspended in fresh 0.32 M sucrose (equal to the volume decanted) and again centrifuged for 25 min at 10.000 *g* and 4°. The pellet from this spin (P'_2) was re-suspended in 0.32 M sucrose (3 ml/g of brain), and the suspension layered on the discontinuous sucrose gradient [8]. Following a 2-hr centrifugation at 65.000 *g* (IEC B-60, rotor Damon SB-110) and 4°, the material at the 0.8–1.2 M sucrose interface (synaptosomes) was removed and diluted with approximately 10 vol. of ice-cold Ca^{2+} free 132 mM NaCl + 5 mM KCl. The fraction thus obtained was not a pure preparation of presynaptic nerve-endings, but it will be referred to as a preparation of synaptosomes in conformity with a number of authors [see ref. 10]. The dilution was made by progressive addition of aliquots (5–10 ml) of the ice-cold Ca^{2+} free 132 mM NaCl + 5 mM KCl over a 15 min period, in order to return the synaptosomes to a more physiological environment. The diluted suspension was then centrifuged at 9000 *g* for 4 min and 4°, and the resulting pellet re-suspended in the 132 mM NaCl + 5 mM KCl + 1.2 mM $CaCl_2$. The re-suspended synaptosomes were then pre-incubated for 12 min at 30° in order to permit them to re-accumulate K^+ and extrude Na^+, and come to a steady state [10]. All centrifugations took place in a IEC B-20 centrifuge, rotor IEC-870.

Protein was determined by the method described by Lowry *et al.* [11], using bovine serum albumin as a standard.

Electron microscopy. The qualitative and quantitative control of the synaptosomes in the preparation was made by electron microscopy. A pellet obtained by sedimentation of the fraction being studied was fixed in 2 per cent OsO_4, and contrasted with 1 per cent APT [8]. This material was included in epon, and thin sections taken from five different blocks were studied under electron microscopy. The observations showed that the fraction contained some "residual" membranes, few mitochondria and some ovoid bodies of variable density and characteristic contents, that were identifiable as synaptosomes. To characterize this subcellular fraction as to its percentage in synaptosomes, stereological techniques were used [12]. The evaluation of the quantity of synaptosomes present was made by determining the percentage fraction of the total volume of structures present, which is occupied by the synaptosomes. The value was obtained by point counting in a series of 10 ultra-micrographs of thin sections obtained from 5 different blocks. Synaptosomes make up ~ 40 per cent of structures present in the subcellular fraction.

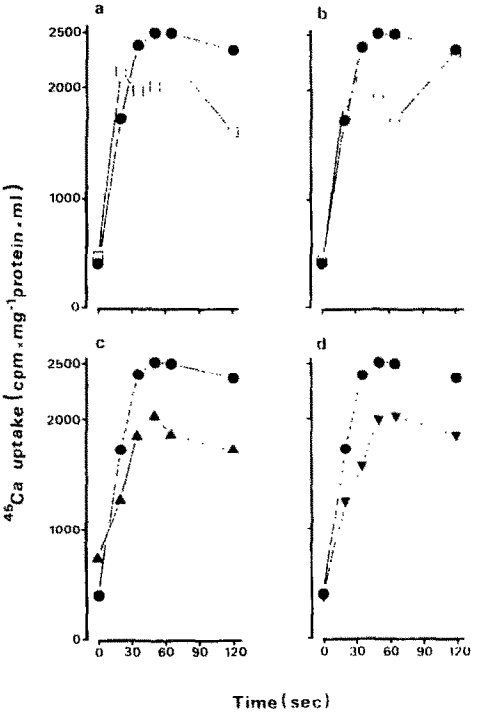

Fig. 1(a–d). Time course of ^{45}Ca uptake by synaptosomes stimulated by K^+ in the absence (●) and in the presence of adenosine 0.1 mM ( ), 0.2 mM (○), 1 mM (▲) and 2 mM (▼).

The uptake of ^{45}Ca by synaptosomes was initiated when 4 ml suspension in 132 mM NaCl + 5 mM KCl + 1.2 mM $CaCl_2$ was incubated with 4 ml stimulator solution (137 mM KCl + 1.2 mM $CaCl_2$ containing ^{45}Ca (0.6 μM). Thus the final solution had 66 mM NaCl + 71 mM KCl + 1.2 mM $CaCl_2$ (specific activity ≃ 0.45 μCi ^{45}Ca per μmol Ca). Adenosine in the different concentrations was present in the respective stimulator solution to test the effect of each concentration on ^{45}Ca uptake. Incubations were carried out at 30°. The uptake of ^{45}Ca was stopped at various time intervals adding 1 ml of the suspension in the stimulator solution to 1 ml of ice-cold EGTA (30 mM) stopping solution. The zero time was determined by the simultaneous addition of 0.5 ml suspension in 132 mM NaCl + 5 mM KCl + 1.2 mM $CaCl_2$ and 1 ml of ice-cold EGTA stopping solution to 0.5 ml stimulator solution (137 mM KCl + 1.2 mM $CaCl_2$) containing ^{45}Ca. The synaptosome suspension was filtered by suction through Millipore filters (0.3 μm) prewashed with ice cold 132 mM choline Cl + 5 mM KCl + 1.2 mM $CaCl_2$ solution. After filtration, each filter was washed with 15 ml ice cold 132 mM choline Cl + 5 mM KCl + 1.2 mM $CaCl_2$. The ^{45}Ca content on the filter was determined by liquid scintillation spectrometry. The counting was made in a Beckman LS 100 C set for maximum counting efficiency for ^{45}Ca. The protein concentration in the suspension was 0.945 mg/ml.

RESULTS AND DISCUSSION

Typical results from an experiment in which were used different concentrations of adenosine are illustrated in Fig. 1. As it can be seen, adenosine (0.1, 0.2, 1 and 2 mM) reduced the uptake of ^{45}Ca by synaptosomes. The preparations were stimulated by high potassium (71 mM) with the purpose of increasing the rate of Ca^{2+} accumulation by synaptosomes [7] and hence simulating the experimental conditions obtained when using high potassium or electrical stimulations of motor nerve endings. All four concentrations of adenosine used reduced the uptake of ^{45}Ca; however, the rate of accumulation decreased when increasing the concentration, and both 1 mM and 2 mM of adenosine induced clearer results than those obtained with lower concentrations (0.1 or 0.2 mM). Figure 2a shows the uptake of

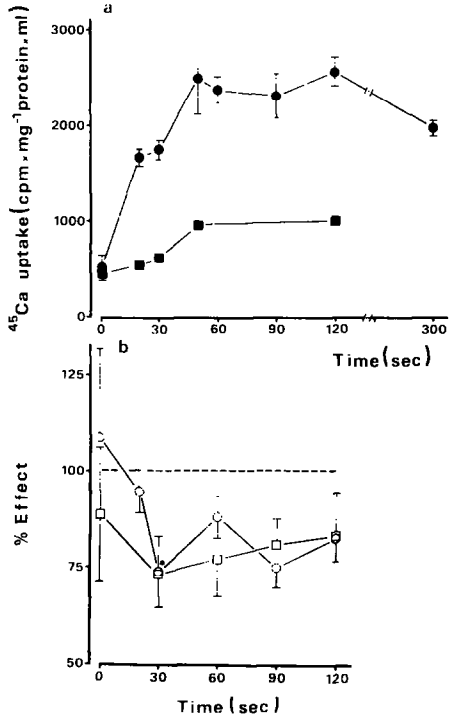

Fig. 2(a). Time course of ^{45}Ca uptake by synaptosomes incubated in 132 mM NaCl + 5 mM KCl + 1.2 mM $CaCl_2$ in one experiment (■) and in 66 mM NaCl + 71 mM KCl + 1.2 mM $CaCl_2$ (● $N = 5$).

The abcissae indicate the periods of incubation. The zero time was determined by the simultaneous addition of 0.5 ml suspension in 132 mM NaCl + 5 mM KCl + 1.2 mM $CaCl_2$ and 1 ml of ice-cold EGTA stopping solution to 0.5 ml stimulator solution (137 mM KCl + 1.2 mM $CaCl_2$) containing ^{45}Ca (0.6–2.4 μM). Thus the final solution had 66 mM NaCl + 71 mM KCl + 1.2 mM $CaCl_2$ (specific activity 0.3–0.6 μCi ^{45}Ca per μmol Ca). (b) Effect of adenosine 0.2 mM ($N = 3$) and of ATP 0.2 mM (○ $N = 5$) on the uptake of ^{45}Ca by synaptosomes. Adenosine and ATP were present in the respective stimulator solutions containing ^{45}Ca. Incubations were carried out at 30°. The broken line is 100% and represents the normal uptake of ^{45}Ca and corresponds to the curve (●) illustrated in a. The vertical bars are ± S.E.M. * $P < 0.05$ (Student's t test). The protein concentration in the suspensions varied between 0.5–1 mg/ml. For further details see caption of Fig. 1.

^{45}Ca by synaptosomes incubated in a solution containing normal potassium (5 mM) in one experiment plotted as a function of time. The uptake was maximum between 30 and 60 sec after starting the incubation, remaining almost at the same level after the next 60 sec. If, however, the synaptosomes were incubated in solutions containing high potassium (71 mM), a new ^{45}Ca uptake process appears—one which is greatly increased by the stimulating action of potassium. Five experiments are illustrated in Fig. 2a (includes the control illustrated in Fig. 1). It is evident that there was a marked increase on the uptake of ^{45}Ca by synaptosomes in all the five experiments in which it was studied. The uptake was linear during the first 30 sec and was maximum also between 30 and 60 sec after starting the incubation, remaining more or less so during the next 4 min in which determinations were made every 30 sec. Figure 2b shows the percentage inhibition of the potassium stimulated ^{45}Ca uptake induced by adenosine (0.2 mM) (includes the experiment illustrated in Fig. 1b). The reduction on the ^{45}Ca uptake was maximum (26.8 + 8 per cent with respect to control) at 30 sec. Similar results were obtained with 0.2 mM ATP in five experiments with a maximum reduction of 26.4 + 9.3 per cent (Fig. 2b) also at 30 sec. However ATP (1 mM) did not change significantly the uptake of ^{45}Ca in two experiments in which it was used, and ATP (2 mM) even increased it in all five experiments in which it was tried, with a maximum increase of 119 + 11.3 per cent at 30 sec. An explanation for this increase could be achieved in terms of a situation similar to that described by Kendrick *et al.* [13], in which ATP (1 mM) promotes the entry of ^{45}Ca into disrupted synaptosomes. Although considered 'morphologically intact', the synaptosomes may not be 'functionally intact' [see ref. 7]. Moreover, it has been shown [13] that 2 mM of ATP increases slightly the uptake of ^{45}Ca by intact synaptosomes, though unstimulated and incubated in a very low Ca^{2+} concentration. A possibility is that ATP as such could cross the synaptosome membranes as it happens in the liver and kidney cells [14]; ATP promotes the uptake of Ca^{2+} by mitochondria [see ref. 15] and by non-mitochondrial structures [13]. In that case, 2 mM of ATP externally applied can cause an effect, since its concentration may be higher than the concentration of ATP estimated as normal in the nerve endings. Such an interpretation, however, does not apply when ATP in a concentration of 0.2 mM was used. More extensive results are required on the effects of ATP either using low or high concentrations as well as in the presence of low or high Ca^{2+} concentrations; however it seems that ATP can have a dual effect: (1) it reduces the permeability of the pre-synaptic membranes to Ca^{2+} [16] by an interaction with a specific protein [17]; and (2) it increases the Ca^{2+} uptake by intra-neuronal structures. In both cases, the physiological importance of ATP as a possible modulator of neurotransmission appears very likely. In fact, it has been suggested [18] that the spontaneous release of the transmitter depends on the resting level of intracellular calcium, which is regulated in most part by the mitochondria and by the non-mitochondrial structures, and that the evoked release of the transmitter depends on the resting level of calcium and the calcium brought inside the nerve endings by depolarization. It happens that ATP applied at the neuromuscular junction reduces

both the evoked and the spontaneous release of the transmitter [3, 19]. The dual effect of ATP presented here (it decreased ^{45}Ca uptake by synaptosomes in low concentrations (0.2 mM) and increased it in high concentrations (2 mM)) also has interest since, for instance, the inhibitory effect of adenosine on neuroeffector transmission of the electrically stimulated longitudinal muscle strip of guinea-pig ileum, recorded as a depression on the isometric contractions, is similar to the inhibitory effect induced by ATP only when low concentrations (up to 0.1 mM) of both compounds were used. At higher concentrations (> 0.1 mM) ATP causes a contraction of the smooth muscle [20]. It follows that these findings have been tentatively explained in terms of Ca^{2+} involvement.

The present results also show that adenosine (0.1–2 mM) decreased the uptake of ^{45}Ca by synaptosomes depolarized by potassium stimulation in the same manner as 0.2 mM of ATP did. Two points seem worth noting in relation to these effects. First, the preparation of synaptosomes we used was a mixture of cholinergic and adrenergic terminals [7]. Secondly the concentrations found as effective concerning the reduction on the ^{45}Ca uptake were similar to those found to inhibit maximally the release of neurotransmitters (Ach and NA) [2, 3, 20–25] and those found to inhibit maximally the postsynaptic excitatory potentials of olfactory cortex slices of the guinea-pigs [4, 5]. Extrapolating the present experimental evidence to these findings, it seems very possible that the inhibitory effects of adenosine and ATP on the evoked release of Ach or NA result from a reduction on the Ca^{2+} entry that follows depolarization of nerve terminals. Thus, assuming that in physiological conditions the release of nucleotides or adenosine occurs at the nerve terminals [26–31], a physiological mediation of the release of neurotransmitters can involve a previous mediation by those substances, of the Ca^{2+} entry emphasizing its physiological role as negative feed-back modulators of the evoked release of neurotransmitters.

In summary, adenosine and ATP decrease the uptake of ^{45}Ca by synaptosomes stimulated by potassium. These results suggest that its inhibitory actions on the release of neurotransmitters are mediated by a reduction on the entry of Ca^{2+} that follows depolarization of the nerve endings.

Acknowledgement We should like to thank Dr. Lucinda R. Mata for her help on electron microscopy.

REFERENCES

1. J. A. Ribeiro, *Life Sci.* **22**, 1373 (1978).
2. B. L. Ginsborg and G. D. S. Hirst, *J. Physiol., Lond.* **224**, 629 (1972).
3. J. A. Ribeiro and J. Walker, *Br. J. Pharmac.* **54**, 213 (1975).
4. Y. Kuroda, M. Saito and K. Kobayashi, *Brain Res.* **109**, 196 (1976).
5. C. N. Scholfield, *Br. J. Pharmac.* **63**, 239 (1978).
6. B. Katz and R. Miledi, *J. Physiol., Lond.* **195**, 481 (1968).
7. M. P. Blaustein, *J. Physiol., Lond.* **247**, 617 (1975).
8. E. G. Gray and V. P. Whittaker, *J. Anat.* **96**, 79 (1962).
9. J. W. Gurd, L. R. Jones, H. R. Mahler and W. J. Moore, *J. Neurochem.* **22**, 281 (1974).
10. M. P. Blaustein and J. M. Goldring, *J. Physiol., Lond.* **247**, 589 (1975).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
12. E. R. Weibel, in *Principles and Techniques of Electron Microscopy* (Ed. M. A. Hayat) Vol. 3, p. 237. Van Nostrand–Reinhold–Company, New York (1973).
13. N. C. Kendrick, M. P. Blaustein, R. C. Fried and R. W. Ratzlaff, *Nature, Lond.* **265**, 246 (1977).
14. I. H. Chaudry, M. M. Sayeed and A. E. Baue, *Can. J. Physiol. Pharmac.* **54**, 742 (1976).
15. A. L. Lehninger, *Biochem. J.* **119**, 129 (1970).
16. M. Weller and I. G. Morgan, *Biochim. biophys. Acta* **465**, 527 (1977).
17. L. G. Abood, K. Kurahasi, E. Gruner and M. P. del Cerro, *Biochim. biophys. Acta* **153**, 531 (1968).
18. E. Alnaes and R. Rahamimoff, *J. Physiol., Lond.* **248**, 285 (1975).
19. J. A. Ribeiro and M. L. Dominguez, *J. Physiol., Paris* **74**, 491 (1978).
20. E. S. Vizi and J. Knoll, *Neuroscience* **1**, 391 (1976).
21. P. Hedqvist and B. B. Fredholm, *Naunyn-Schmiedeberg's Arch. Pharmac.* **293**, 217 (1976).
22. M. A. Enero and B. Q. Saidman, *Naunyn-Schmiedeberg's Arch. Pharmac.* **297**, 39 (1977).
23. R. H. Verhaeghe, P. M. Vanhoutte and J. T. Shepherd, *Circ. Res.* **40**, 208 (1977).
24. C. Su, *J. Pharmac. exp. Ther.* **204**, 351 (1978).
25. A. R. Wakade and T. D. Wakade, *J. Physiol., Lond.* **282**, 35 (1978).
26. G. Burnstock, G. Campbell, D. Satchell and A. Smythe, *Br. J. Pharmac.* **40**, 668 (1970).
27. C. Su, J. A. Bevan and G. Burnstock, *Science* **173**, 336 (1971).
28. I. Pull and H. McIlwain, *Biochem. J.* **130**, 975 (1972).
29. E. M. Silinsky, *J. Physiol., Lond.* **247**, 145 (1975).
30. P. V. Sulakhe and J. W. Phillis, *Life Sci.* **17**, 551 (1975).
31. B. B. Fredholm, *Acta physiol. scand.* **96**, 422 (1976).