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# Endogenous Adenosine Modulation of $^{22}Na$ Uptake by Rat Brain Synaptosomes*

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To evaluate if endogenous extracellular adenosine influences sodium channel activity in nerve terminals, we investigated how manipulations of extracellular adenosine levels influence $^{22}Na$ uptake by rat brain synaptosomes stimulated with veratridine (VT). To decrease extracellular adenosine levels, adenosine deaminase (ADA) that converts adenosine into an inactive metabolite was used. To increase extracellular adenosine levels, we used the adenosine deaminase inhibitor erythro-9(2-hydroxy-3-nonyl) adenine (EHNA), as well as the inhibitor of adenosine transport, nitrobenzylthioinosine (NBTI). ADA (0.1–5 U/ml) caused an excitatory effect on $^{22}Na$ uptake stimulated by veratridine, which was abolished in the presence of the adenosine deaminase inhibitor erythro-9(2-hydroxy-3-nonyl) adenine (EHNA, 25 μM). Both the adenosine uptake inhibitor nitrobenzylthioinosine (NBTI, 1–10 μM) and the adenosine deaminase inhibitor EHNA (10–25 μM) inhibited $^{22}Na$ uptake by rat brain synaptosomes. It is suggested that adenosine is tonically inhibiting sodium uptake by rat brain synaptosomes.

**KEY WORDS:** Adenosine; $^{22}Na$ uptake; synaptosomes; adenosine deaminase; adenosine uptake inhibition.

## INTRODUCTION

In 1977 one of us (J. A. Ribeiro) discussed with Arsélio the role of presynaptic calcium in adenosine modulation of neurotransmitter release. Previous results suggested that calcium might be involved in this effect (1), but it was very difficult to distinguish in electrophysiological studies whether those substances could modify calcium entry into stimulated nerve endings. Arsélio suggested using as a preparation rat brain synaptosomes stimulated by potassium and studying $^{45}Ca^{2+}$ uptake. This suggestion introduced our group routinely to the synaptosomal preparation and since our first publication in *Biochemical Pharmacology* (2), our group published 23 original papers using synaptosomes as a way to answer questions on presynaptic mechanisms. In 1983 Ana Sebastião discussed with Arsélio, who introduced her to the Neurochemistry courses at the Gulbenkian Institute of Science in Oeiras, the possibility of exploring $^{22}Na$ uptake by synaptosomes stimulated by veratridine as a way to know how purines might modulate nerve conduction (3–4).

The last original work performed by our group using synaptosomes is now presented as a way to honor and to express our admiration for Arsélio, a guide of science for all of us, since his move from the United States in early 1970s to develop his scientific career at the University of Coimbra.

It has been described that some substances block epileptiform activity by associating their capacity to increase endogenous adenosine, probably by inhibition of adenosine deaminase, with the ability to directly inhibit voltage gated sodium channels (see e.g., [5]). It is

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known that adenosine analogues by activating a xanthine-sensitive adenosine receptor (6), inhibit sodium uptake by rat brain synaptosomes stimulated by veratridine (VT), an effect mediated through tetrodotoxin (TTX)-sensitive sodium channels (4,6). As previously discussed, the action of adenosine might be related to inhibition of the voltage-gating system of the sodium channels (4) as a result of an enhancement of the intrasynaptosomal cyclic AMP concentrations (6).

Under basal conditions, synaptosomes from the rat brain can release adenosine as such or this nucleoside can originate from the metabolism of released nucleotides, probably ATP. Released ATP is metabolized into adenosine by ecto-ATPases (7) that convert ATP into ADP and ADP is metabolized to AMP by ADPases. AMP is converted to adenosine by the enzyme ecto-5′-nucleotidase (8). Extracellular adenosine accumulation may also be induced by depolarization of synaptosomes with high $K^+$ or veratridine (9). Extracellular adenosine levels are regulated by uptake into cells and by metabolism via the actions of extracellular adenosine deaminase or intracellular adenosine kinase (10). It therefore appeared of interest to investigate whether endogenous adenosine modulates $^{22}Na$ uptake by rat brain synaptosomes. This was studied by incubating synaptosomes with the enzyme that hydrolyses adenosine into inactive metabolites, adenosine deaminase (ADA), as well as with the adenosine deaminase inhibitor erythro-9(2-hydroxy-3-nonyl) adenine (EHNA). The action of the adenosine uptake inhibitor nitrobenzylthioinosine (NBTI) was also investigated.

## EXPERIMENTAL PROCEDURE

*Preparation of Synaptosomal Fraction.* A synaptosomal fraction from rat brains without the cerebella was prepared as previously described (6). Briefly, three to four Wistar rats per experiment (about 40 days old) were decapitated and the brains rapidly removed into ice-cold 0.32 M sucrose buffered with Tris 20 mM to pH 7.4, homogenized in 9 volumes of 0.32 M sucrose and centrifuged (0–4°C) at 1500 *g* for 10 min. The supernatant was kept and the sediment resuspended and centrifuged at the same speed and time. The supernatants obtained from both centrifugations were mixed and centrifuged at 9000 *g* for 20 min. After washing, the pellet was dispersed in 10 ml of 0.32 M of sucrose buffered with Tris 20 mM. Five-milliliter aliquots of the suspension were layered carefully on 20 ml of 0.8 M sucrose buffered with Tris 20 mM (pH 7.4) and centrifuged at 9000 *g* for 30 min. After centrifugation, the fraction retained at the interface of 0.8 M sucrose (synaptosomal fraction) was collected and its concentration adjusted to 0.4 M of sucrose by dilution with Tris 20 mM (pH 7.4). After centrifugation at 20 000 *g* for 30 min, the pellet was resuspended in a sodium-free solution containing 130 mM choline chloride, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, and 50 mM HEPES-Tris 50 (pH 7.4) and 1 mg/ml of bovine serum albumine (BSA). Protein concentration was determined (11), and its value was 7.49 ± 0.72 mg/ml.

*Determination of $^{22}Na$ Uptake by Synaptosomes.* Sodium uptake through the sodium channels was measured (12) and modified according to a previous report (4). Samples (45 μl) of synaptosomal suspension plus 5 μl of the test drugs or resuspension medium were preincubated at 36°C for 10 min and with VT (20 μM) for 1 min followed by incubation with the $^{22}Na$ (1 μCi/ml) containing solutions (150 μl) for 5 s. The incubation solutions contained besides the desired concentrations of VT and the test drugs. 127 mM choline chloride, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, 50 mM HEPES Tris 50 (pH 7.4), 2.8 mM NaCl 1 mg/ml of bovine serum albumine, 1.4 μCi/ml carrier-free $^{22}NaCl$ (specific activity in the working solution ≈0.5 μCi/μM), and 5 mM of ouabain. Incubation was terminated by adding 3 ml of ice-cold wash solution (163 mM of choline chloride, 0.8 mM $MgSO_4$, 1.4 mM $CaCl_2$, 5 mM HEPES-Tris (pH 7.4) and 1 mg/ml BSA) followed by filtration under reduced pressure through 0.45-μm cellulose filters, and the $^{22}Na$ trapped on the filters was counted in a scintillation counter. Nonspecific $^{22}Na$ uptake was determined using tetrodotoxin (1 μM) in the VT-containing solutions, and the data are presented as correct specific $^{22}Na$ uptake after subtraction of nonspecific uptake from total VT-induced $^{22}Na$ uptake. The specific $^{22}Na$ uptake was 2.89 ± 0.65 nM/mg of protein and significantly different ($P < .05$, $n = 56$) from nonspecific $^{22}Na$ uptake, which was 0.46 ± 0.09 nM/mg of protein. In each experiment, $^{22}Na$ uptake determinations were performed in triplicate. In each experiment the $^{22}Na$ uptake was determined in the absence (control) and in the presence of the test drugs. When testing the ability of EHNA to modify actions of ADA, EHNA was present both in control and in test tubes

*Statistics.* Data are expressed as mean ± SEM. P values of .05 or less (Student's t test) were considered to represent significant differences.

*Drugs.* Drugs used were tetrodotoxin (TTX), ouabain, adenosine deaminase (type VI, 1782.2 U/ml, EC 3.5.4.4), veratridine (VT), *S*-(*p*-nitrobenzyl)-6-thioinosine (NBTI) (Sigma), and erythro-9(2-hydroxy-3-nonyl)adenine (EHNA) (Boehringer). Carrier-free $^{22}NaCl$ (specific activity of purchased stock solution, ≈11 mCi/mM) was from Amersham. All other chemicals and solvents were obtained from common commercial sources.

## RESULTS

### Tonic Adenosine Modulation of $^{22}Na$ Uptake in Rat Brain Synaptosomes

The action of exogenous adenosine deaminase (ADA) on VT-stimulated $^{22}Na$ uptake by synaptosomes is shown in Fig. 1. As can be seen, the effect of ADA on $^{22}Na$ uptake was enhanced, as the amount of ADA in preincubation medium was increased from 0.1 to 2.5 U/ml. The averaged increase in the sodium uptake caused by ADA (2.5 U/ml). When ADA (2.5 U/ml) was incubated with synaptosomes in the presence of the adenosine deaminase inhibitor erythro-9(2-hydroxy-3-nonyl)adenine (EHNA), the excitatory effect of ADA on

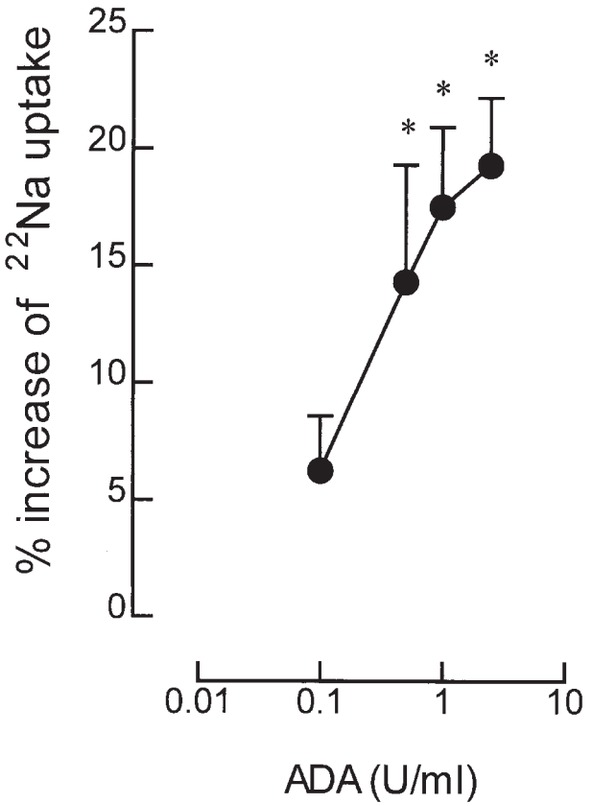


**Fig. 1.** Effect of adenosine deaminase (ADA) on specific $^{22}Na$ uptake by rat brain synaptosomes stimulated by VT. Incubation of the synaptosomal fraction (containing 0.28–0.42 mg of protein) with $^{22}Na$ was performed for 5 s. The ordinate represents the percentage modification on veratridine-induced specific $^{22}Na$ uptake; 0% is the specific $^{22}Na$ uptake in the same experiment but in the absence of ADA (2.27 ± 0.17 nM/mg of protein) and 100% represents a complete inhibition of $^{22}Na$ uptake. Averaged nonspecific $^{22}Na$ uptake observed in the presence of VT (20 μM) + TTX (1 μM) in the same experiments was 0.45 ± 0.08 nM/mg of protein. * $P < .05$ compared with control. Means + SEM from eight experiments; in each experiment the assays were performed in triplicate.

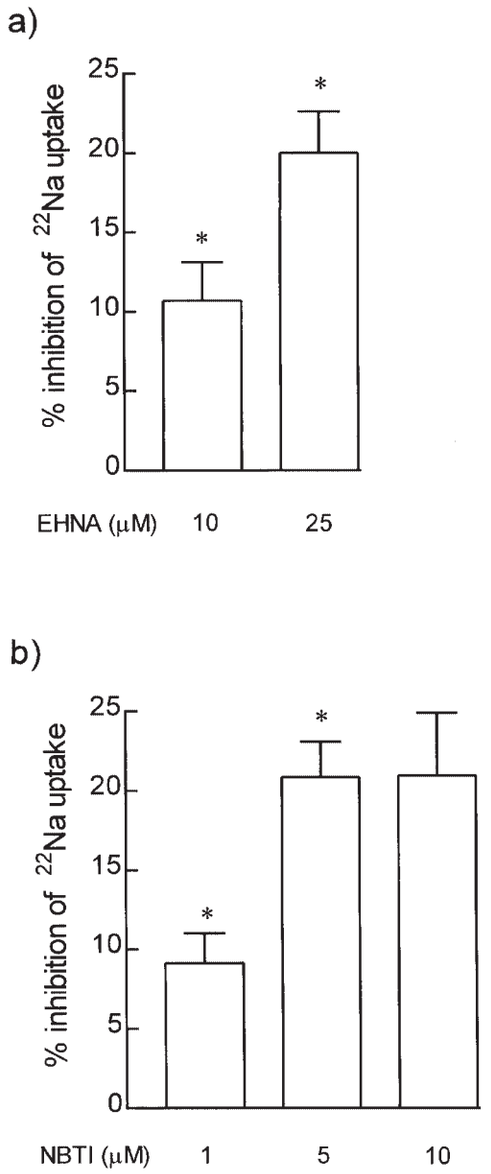


**Fig. 2.** Influence of inhibition of extracellular adenosine inactivation by erythro-9(2-hydroxy-3-nonyl)adenine (EHNA) (a) and *S*-(*p*-nitrobenzyl)-6-thioinosine (NBTI) (b) on VT-induced specific $^{22}Na$ uptake by rat brain synaptosomes. Synaptosomal fractions contained between 0.30–0.44 mg of protein. Synaptosomes were preincubated with the test drugs for 10 min and with VT (20 μM) for 1 min before addition of the $^{22}Na$-containing solutions. Incubation of the synaptosomal fraction with $^{22}Na$ (specific activity 0.5 μCi/μM of $Na^+$) was performed for 5 s. The ordinate represents the percentage inhibition of veratridine-induced specific $^{22}Na$ uptake. (a) 0% (control) was 3.35 ± 0.30 nM/mg of protein and averaged nonspecific $^{22}Na$ uptake in the same experiments was 0.70 ± 0.11 nM/mg of protein. Means + SEM of eight experiments for each bar. * $P < .05$ compared with control; (b) 0% was 3.58 ± 0.27 and nonspecific $^{22}Na$ uptake in the same experiments was 0.82 ± 0.08 nM/mg of protein. Means + SEM of nine experiments, except in the case of 10 μM NBTI (n = 2). * $P < .05$ compared with control. In each experiment the assays were performed in triplicate.

$^{22}Na$ uptake was attenuated 85% by 10 μM EHNA, and 25 μM of EHNA even completely abolished the excitatory effect of ADA (n = 2).

### Effects of Erythro-9(2-Hydroxy-3-Nonyl) Adenine and *S*-(*p*-Nitrobenzyl)-6-Thioinosine on $^{22}Na$ Uptake by Synaptosomes.

Figure 2 illustrates the effect of the adenosine deaminase inhibitor EHNA (10 and 25 μM) on VT-induced $^{22}Na$ uptake by brain synaptosomes. EHNA decreased $^{22}Na$ uptake; the inhibitory effect obtained with 25 μM

was 19.7 ± 2.6%, (n = 9, P < .05), which was more pronounced than that obtained with 10 μM (10.4 ± 2.4%, n = 9, P < .05).

As happened with EHNA, the adenosine uptake inhibitor NBTI (1–10 μM) also decreased $^{22}$Na uptake. The effects of 5 and 10 μM were similar (20.5 ± 2.2%, n = 9, and 20.7 ± 4.0%, n = 2), and both were greater than the inhibitory effect of a lower concentration (1 μM) of NBTI (8.9 ± 1.9%, n = 9, P < .05).

## DISCUSSION

The present results show that substances that can modify the extracellular levels of adenosine modulate $^{22}$Na uptake by rat brain synaptosomes stimulated by veratridine, because adenosine deaminase, the enzyme that hydrolyzes adenosine into inactive metabolites, increased $^{22}$Na uptake, and the adenosine deaminase inhibitor, EHNA as well as the adenosine uptake blocker NBTI, inhibited $^{22}$Na uptake. The release of neurotransmitter from nerve endings is very sensitive to small changes in membrane potential, and small changes in sodium channel function may cause changes in the threshold for the action potential generation. Therefore, small changes in sodium uptake may have physiologically significant actions on neuronal function. In the present work, the excitatory effects induced by adenosine deaminase on $^{22}$Na uptake, were about 19%, suggesting the presence of a discrete effect of endogenous adenosine tonically inhibiting $^{22}$Na uptake. Although small, it may influence membrane potential and the threshold for action potential generation, thus affecting neuronal excitability.

It has been reported (13) that adenosine deaminase might have neuronal actions not related to its ability to remove extracellular adenosine, but the present observation that an inhibitor of adenosine deaminase, EHNA, prevented the excitatory effect of ADA on $^{22}$Na uptake by synaptosomes, suggests that this effect is due to its ability to remove endogenous adenosine, which tonically inhibited $^{22}$Na uptake.

[$^{3}$H]NBTI binds to synaptosomes and inhibits the cotransport of nucleosides, therefore increasing extracellular adenosine wherever extracellular adenosine is higher than intracellular concentration (14). NBTI inhibited $^{22}$Na uptake by rat brain synaptosomes, suggesting that there is an increase in extracellular adenosine due to the inhibition of the NBTI-sensitive nucleoside transporter. One can argue that the inhibitory effect of NBTI on $^{22}$Na uptake by synaptosomes is not due to an increase of extracellular adenosine with subsequent inhibition of voltage-dependent sodium channels, but a result of inhibition of an NBTI-sensitive sodium transport. This is probably not the case because NBTI did not influence the sodium transport, which is not mediated by voltage-dependent sodium channels, that is, that occurred in the presence of a supramaximal concentration of TTX. Furthermore, as occurred with NBTI, the adenosine deaminase inhibitor EHNA also inhibited $^{22}$Na uptake. Although adenosine deaminase has been considered to be cytosolic (but see [10]), there have been some reports of a membrane-associated adenosine deaminase in rat brain subfractions (15) and inhibition of adenosine deaminase enhances presynaptic actions of adenosine in the rat phrenic nerve (16). However, EHNA can also be taken up by purine transporters and inhibit intracellular adenosine deaminase, with subsequent enhancement of adenosine release.

Some drugs used in the treatment of brain dysfunctions, such as benzodiazepines (17) and phenothiazines (18), interfere with mechanisms of inactivation of endogenous adenosine, increasing its extrasynaptosomal levels. Endogenous adenosine plays an important role in neuronal excitability, especially in the hippocampus (e.g., [19]). Adenosine and some of its analogues possess anticonvulsant actions (see e.g., [20]). On the other hand, the antiepileptic agents diphenylhydantoin and carbamazepine, at therapeutic concentrations, bind to voltage-dependent sodium channels (21). The existence of a tonic modulation by adenosine of sodium uptake by rat brain synaptosomes, together with the well-known presynaptic and postsynaptic modulatory effects of adenosine (see e.g., [22]), support the idea of multiple mechanisms underlying the inhibitory action of the nucleoside on neuronal excitability, reinforcing its role as an endogenous anticonvulsant (23).

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## REFERENCES

1. Ribeiro, J. A. and Walker, J. 1975. The effects of adenosine triphosphate and adenosine diphosphate on transmission at the rat and frog neuromuscular junctions. Br. J. Pharmacol. 54:213–218.
2. Ribeiro, J. A., Sa-Almeida, A. M., and Namorado, J. M. 1979. Adenosine and adenosine triphosphate decrease $^{45}$Ca uptake by

synaptosomes stimulated by potassium. Biochem. Pharmacol. 28:1297–1300.
3. Ribeiro, J. A. and Sebastião, A. M. 1984. Antagonism of tetrodotoxin-and procaine-induced axonal blockade by adenine nucleotides in the frog sciatic nerve. Br. J. Pharmacol. 81: 277–282.
4. Simões, A. P., Oliveira, P. C., Sebastião, A. M., and Ribeiro, J. A. 1988. $N^6$-Cyclohexyladenosine inhibits veratridine-stimulated $^{22}Na$ uptake by rat brain synaptosomes. J. Neurochem. 50: 899–903.
5. Dupere, J. R., Dale, T. J., Starkey, S. J., and Xie, X. 1999. The anticonvulsant BW534U87 depresses epileptiform activity in rat hippocampal slices by an adenosine-dependent mechanism and through inhibition of voltage-gated $Na^+$ channels. Br. J. Pharmacol. 128:1011–1020.
6. Lobo, M. G. and Ribeiro, J. A. 1992. Effects of forskolin, dibutyryl cyclic AMP and 5′-*N*-ethylcarboxamide adenosine on $^{22}Na$ uptake by rat brain synaptosomes stimulated by veratridine. J. Neurochem. 58:1033–1037.
7. Nagy, A. 1986. Enzymatic characteristics and possible role of synaptosomal ecto-adenosine triphosphatase from mammalian brain. Pages 49–59, in Kreutzberg et al. (eds.) Cellular Biology of Ectoenzymes, Spinger-Verlag, Berlin Heidelberg.
8. Sebastião, A. M., Cascalheira, J. F., Cunha, R. A., and Ribeiro, J. A. 1999. Adenine nucleotides as inhibitors of synaptic transmission: Role of localised ectonucleotidases. Prog. Brain Res. 120:183–192.
9. MacDonald, W. F. and White, T. D. 1985. Nature of extrasynaptosomal accumulation of endogenous adenosine evoked by $K^+$ and veratridine. J. Neurochem. 45:791–797.
10. Meghji, P. 1991. Adenosine production and metabolism. Pages 25–42, in Stone, T. W. (ed.) Adenosine in the Central Nervous System, Academic Press, London.
11. Lowry, H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
12. Tamkun, M. M. and Catterall, W. A. 1981. Ion flux studies of voltage sensitive sodium channels in synaptic nerve-ending particles. Mol. Pharmacol. 19:78–86.
13. Catania, M. V., Sortino, M. A., Rampello, O. L., Canonico, P. L., and Nicoletti, F. 1991. Adenosine deaminase increases release of excitatory amino acids through a mechanism independent of adenosine depletion. Neuropharmacology 30:153–159.
14. Marangos, R. D., Patek, J., Rosenberg, C., and Marino, A. M. 1982. [$^3H$] Nitrobenzylthioinosine binding as a probe for the study of adenosine uptake sites in brain. J. Neurochem. 39:184–191.
15. Franco, R., Casadó, V., Ciruela, F., Saura, C., Mallol, J., Canela, E., and Lluis, C. 1997. Cell surface adenosine deaminase: Much more than an ectoenzyme. Prog. Neurobiol. 52:283–294.
16. Sebastião, A. M. and Ribeiro, J. A. 1988. On the adenosine receptor and adenosine inactivation at the rat diaphragm neuromuscular junction. Br. J. Pharmacol. 94:109–120.
17. Phillis, J. W. Bender, A. S., and Wu, P. H. 1980. Benzodiazepines inhibit adenosine uptake into rat brain synaptosomes. Brain Res. 195:494–498.
18. Phillis, J. W. and Wu, P. H. 1981. Phenothiazines inhibit adenosine uptake by rat brain synaptosomes. Can. J. Physiol. Pharmacol. 59:1108–1110.
19. Mitchell J. B., Lupica C. R., and Dunwiddie T. V. 1993. Activity-dependent release of endogenous adenosine modulates synaptic responses in the rat hippocampus. J. Neurosci. 13:439–3447.
20. Dragunow, M. 1986. Adenosine: The brain's natural anticonvulsant? Trends Pharmacol. Sci. 7:128–130.
21. Willow, M. 1986. Pharmacology of diphenylhydantoin and carbamazepine action on voltage-sensitive sodium channels. Trends Pharmacol. Sci. 4:147–149.
22. Sebastião, A. M. and Ribeiro, J. A. 2000. Fine tuning neuromodulation by adenosine. Trends Pharmacol. Sci. 21:341–346.
23. Ribeiro, J. A. and Sebastião, A. M. 1987. Adenosine, cyclic AMP and nerve conduction. Pages 559–573, in Gerlach, E. and Becker, B. F. (eds.), Topics and Perspectives in Adenosine Research, Springer-Verlag Berlin Heidelberg.