References

6. Tomassello F, Mariani F, Fieschi C et al: Assessment of Inter-

Demonstration of Adenosine Receptors on Mouse Cerebral Smooth Muscle Membranes

DAVID W. BECK, M.D., HARRY V. VINTERS, M.D., STEVEN A. MOORE, M.D., PH.D., MICHAEL N. HART, M.D., FRITZ A. HENN, M.D., PH.D., PASQUALE A. CANCELLA, M.D.

SUMMARY Adenosine receptors have been identified on brain cortical membranes and microvascular preparations. However, they have not been demonstrated on specific microvascular elements in isolation. 2-H-chloroadenosine was used as a ligand to investigate the presence of adenosine receptors on isolated mouse cerebral smooth muscle membranes. The binding studies reveal the presence of a high affinity binding site with a Kd value of 33.3 nM and a maximal binding capacity (Bmax) of 283 fmol/mg protein. These findings demonstrate that there is an adenosine receptor on cerebral smooth muscle membranes.

ADENOSINE has been proposed as a neuroregulator of cerebral blood flow because of the rapid rise in brain adenosine levels following hypotension, ischemia, seizures, and hypoxia and because adenosine is a potent vasodilator. In its proposed role of regulating cerebral blood flow it is speculated that adenosine may be produced at the glial foot process via 5'-nucleotidase, released into the extracellular space, and bound to cerebral smooth muscle membranes resulting in relaxation of the vessel. There is some evidence to support this hypothesis. Adenosine receptors A1 and A2 have been identified on brain membranes and are associated respectively with a decrease and an increase in cyclic AMP production. Furthermore, increased smooth muscle cyclic AMP has been associated with vessel relaxation. Adenosine receptors on cerebral smooth muscle membranes, however, have never been demonstrated. Palmer, et al have shown adenosine receptors in capillaries of rat cerebral cortex coupled to adenylate cyclase. However, these microvessel preparations are impure, in that they contain several microvascular elements such as glia, endothelia, smooth muscle, and pericytes. If indeed adenosine regulates cerebral blood flow via action on cerebral smooth muscle membranes, then receptors on specific microvascular elements need to be demonstrated in isolation. We report the results of binding studies of 2-H-chloroadenosine with isolated mouse cerebral smooth muscle membranes. Our results show a high affinity receptor for 2-H-chloroadenosine is present on cerebral smooth muscle membranes.

Methods

Cell Cultures

Isolation of mouse cerebral microvessels and deprivation of cerebral smooth muscle cells and cerebral endothelial cells in tissue culture have previously been described from our laboratory. Cerebral smooth muscle cells are characterized by their broad, polygonally shaped morphology and possess many characteris-
tistics of smooth muscle: basal laminae, clusters of pinocytotic vesicles, and bundles of thin filaments. Isolec-toic focusing and SDS electrophoresis of cellular proteins on polyacrylamide gels after pulse labeling the cultures with [S-35]-methionine demonstrates that these cells actively synthesize α-actin, a smooth muscle specific isoactin. No cells in our cultures stained with either anti-factor VIII antibody, an endothelial specific antibody, or anti-GFAP, an astrocyte specific antibody. It is therefore doubtful that our cultures were contaminated with other brain cell types. Stock cultures of the cell lines were maintained in modified Lewis media (MLM) supplemented with 20% fetal bovine serum and were passed twice weekly. All studies were performed on cells prior to passage 10.

Preparation of membranes

Cells were grown to confluence in 100 mm tissue culture dishes, scraped from the plastic with a "rubber policeman", and placed into centrifuge tubes with Tris-HCl buffer solution (pH 7.4, consisting of 50 mM Tris-HCl, 25 mM MgCl2 and 25 mM CaCl2). Each policeman, and placed into centrifuge tubes with T-culture dishes, scraped from the plastic with a "rubber policeman", and placed into centrifuge tubes with Tris-HCl buffer solution (pH 7.4, consisting of 50 mM Tris-HCl, 25 mM MgCl2 and 25 mM CaCl2). Each experiment required cells from 20 dishes. The cells were disrupted in a Polytron (setting 5) for 30 seconds, and centrifuged at 30,000 × g for 10 minutes. The pellet was then frozen at -20°C until use. After thawing, the pellet was resuspended in 50 mM Tris-HCl buffer containing adenosine deaminase (Sigma type III, 2 I.U./ml) to destroy endogenous adenosine. This was incubated for 30 minutes at 37°. Following incubation the homogenate was washed twice with 50 mM Tris-HCl and resuspended.

The incubation mixture for binding contained 50 μl of homogenate (approximately 0.3–0.8 mg protein) in 50 mM Tris-HCl buffer solution, 10 μl of 2-3H-chloroadenosine (10–12 Ci/mMol, Moravek Biochemicals) and 940 μl Tris-HCl to a final volume of 1.0 ml. For determination of nonspecific binding, 10 μl of 2-3H-chloroadenosine (10–12 Ci/mMol, Moravek Biochemicals) and 930 μl Tris-HCl buffer and 10 μl of 2-3H-chloroadenosine was added to 50 μl of homogenate along with 930 μl Tris-HCl buffer and 10 μl of 2-3H-chloroadenosine. The incubation was carried out at 0–4° for 30 minutes. At the end of the incubation 5 ml of ice cold buffer was added to the sample tube, and the sample was poured onto a 25 mm Whatman 6F/B filter under reduced pressure. The filter was then washed twice with 5 ml ice cold buffer. The radioactivity was measured on a Beckmann LS 7800. Protein content was determined by the Lowry protein assay.

Results

The results presented in figure 1a showed that specific binding of 2-3H-chloroadenosine to cerebral smooth muscle membranes appeared to be saturable when binding was measured at increasing concentrations (0–30 nM). Specific binding was also linear from 0.1 to 1.0 mg of membrane protein (data not shown). Analysis of the data by a Scatchard plot (figure 1b) indicates a linear plot with an apparent dissociation constant (Kd) of 33.3 nM and a maximum binding capacity (Bmax) of 283 fmol/mg protein. Nonspecific binding was less than 20% of the total binding.

Discussion

Palmer et al.13 have shown the presence of adenosine receptors coupled to adenylyl cyclase activity in brain microvessel fractions using 2-chloroadenosine as a ligand. However, these preparations are impure, including elements such as pericytes, endothelium, smooth muscle, and glia. We have demonstrated a high affinity adenosine receptor for 2-3H-chloroadenosine on cerebral smooth muscle membranes with a Kd (33.3 nM) and Bmax (283 fmol/mg protein). This binding is in close agreement with that reported by Wu and Philip21,22 and 2-3H-chloroadenosine binding onto rat cerebral cortical synaptosomal membranes (Kd = 23.5 nM). Williams and Risley21,22 reported that 2-3H-chloroadenosine bound to deaminase treated rat brain membranes at two high affinity sites with Kd values of 1.31 nM and 16.2 nM. Our data is in agreement with the lower of the two (16.2 nM) affinities. The binding of 2-3H-chloroadenosine to microvessel fractions (Kd = 0.038 nM) is a higher affinity binding and may represent the first binding site (Kd = 1.31 nM) reported by Williams and Risley. There are at least two extracellular adenosine receptors associated with adenylyl cyclase activity, and it is unclear whether 2-3H-chloroadenosine binds to the adenosine A1-receptor, associated with a decrease in cyclic AMP accumulation, or the adenosine A2-receptor, associated with an increase in cyclic AMP accumulation.19,20

Adenosine, a potent vasodilator, has been proposed as a neuroregulator of cerebral blood flow via action on cerebral smooth muscle membranes.1,2 Our findings of a high affinity adenosine receptor on isolated cerebral

![Figure 1a. Bindings of 2-3H-chloroadenosine to mouse cerebral smooth muscle membrane. The points are the mean of triplicate determinations of three separate experiments with SD indicated by vertical bars. b. Scatchard plot of (a). The line was generated by linear regression (r = 0.96) yielding values for Kd = 33.3 nM and Bmax = 283 fmol/mg protein.](image-url)
smooth muscle membranes are consistent with this hypothesis. The action of adenosine with the brain microvasculature is undoubtedly complex and its interaction with specific microvascular elements is unclear. It has been shown that adenosine is taken up by isolated microvessels, cerebral endothelium and cerebral smooth muscle via a carrier-mediated system. Also, the affinity of uptake into isolated cerebral endothelium (Km = 5.0 μM) is greater than that into isolated cerebral smooth muscle cells (Km = 10.0 μM). These data suggest that cerebral endothelium may serve to regulate extracellular adenosine concentrations while cerebral smooth muscle serves a more complex role: adenosine may bind to its membrane receptor, possibly resulting in increased intracellular cyclic AMP and vessel relaxation.

Conclusion

We have demonstrated a high affinity adenosine receptor on cerebral smooth muscle membranes. These results are consistent with the hypothesis that adenosine regulates cerebral blood flow via action on cerebral smooth muscle.

Acknowledgements

The authors wish to thank Elizabeth Yoder and Karen Hansen for their expert assistance.

References

11. VanCalker D, Møller M, Hemptich B. Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. J Neurochem 33: 999-1005, 1979
Demonstration of adenosine receptors on mouse cerebral smooth muscle membranes.
D W Beck, H V Vinters, S A Moore, M N Hart, F A Henn and P A Cancilla

*Stroke*. 1984;15:725-727
doi: 10.1161/01.STR.15.4.725

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1984 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/15/4/725

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Stroke* is online at:
http://stroke.ahajournals.org//subscriptions/