

tect *sor* in as large amounts as gp120 and gp41 (in either infected cells or virions). Alternatively, *sor* may participate in the early events in viral replication (from penetration to synthesis of proviral DNA) or in potentiating the cellular environment in which replication occurs. Further studies will be necessary to evaluate these possibilities.

ground levels; (iii) virus particles were detected by electron microscopy.

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20. The presence of each mutation within the new constructs was verified by Southern blotting analysis using the oligonucleotides as probes and in conditions in which the mutated and nonmutated genomes could be distinguished. Plasmids ΔS , 3.3, 6.9, 153, and X, and the parental clone pHXB2gpt were transfected into OKT4⁺ H9 cells by the protoplast fusion approach (7). The transfected cultures were maintained in RPMI 1640 medium containing 20% fetal calf serum and antibiotics for 4 to 6 weeks and analyzed weekly for the presence of HIV-1 *gag* (p17, p24), envelope (p41), and virus particles. HIV-1 *gag* and *env* expression was visualized by using the monoclonal antibodies BT2, BT3, and M25 [F. d. M. Veronese *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5199 (1985)], fluorescein conjugated sheep antibody to mouse immunoglobulin, and standard immunofluorescence approaches. The RT assays were performed on tenfold concentrated spent culture medium by routine procedures (11) with Mg²⁺ being used as a cofactor. Cultures were judged to be HIV-1 infected if they satisfied any of the following criteria: (i) HIV-1 *gag/env* expression could be reproducibly detected in cultures at frequencies exceeding 0.1%; (ii) RT assays performed on at least two sequential samples had values for ³HdTMP incorporated, three or more times back-

A Parathyroid Hormone-Related Protein Implicated in Malignant Hypercalcemia: Cloning and Expression

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Humoral hypercalcemia of malignancy is a common complication of lung and certain other cancers. The hypercalcemia results from the actions of tumor factors on bone and kidney. We report here the isolation of full-length complementary DNA clones of a putative hypercalcemia factor, and the expression from the cloned DNA of the active protein in mammalian cells. The clones encode a prepro peptide of 36 amino acids and a mature protein of 141 amino acids that has significant homology with parathyroid hormone in the amino-terminal region. This previously unrecognized hormone may be important in normal as well as abnormal calcium metabolism.

HYPERCALCEMIA IS FREQUENTLY associated with malignant disease. Humoral hypercalcemia of malignancy (HHM) occurs in cancer patients without bony metastases often in association with squamous cell carcinoma of the lung, where it is a major contributor to morbidity and complicates clinical management (1-3). The hypercalcemia is caused by tumor products acting on bone to promote resorption and on the kidney to restrict calcium excretion (2-4). The biochemical similarities between primary hyperparathyroidism and the HHM syndrome (2, 5) pointed to the likelihood that these tumors produce a substance that has actions very similar to parathyroid hormone (PTH). This tumor factor is distinct from PTH, however, since PTH radioimmunoassays usually fail to detect increased levels of the hormone in plasma from HHM subjects (2, 3, 6) and since PTH messenger RNA is not found in the tumors of such patients (7). Using a bioassay based on the stimulation of adenosine 3',5'-monophosphate (cAMP) levels in the PTH-responsive rat osteogenic sarcoma cell line UMR106-01, we have recently purified a protein of M_r 18,000 from the conditioned medium of a human lung cancer cell line (BEN) derived originally from a patient with HHM syndrome (8). Similar or identical biological activities have also been identified in extracts of tumors from HHM patients (9, 10), from animal tumor models of

the syndrome (10, 11), and from conditioned media from cultures of two such tumors (8, 12). The amino acid sequence of the first 16 residues of the BEN cell-derived protein has been determined, and 8 of the 16 residues are identical with human PTH (8). We describe here the isolation of complementary DNA (cDNA) clones, the complete primary structure, and the active expression in mammalian cells of the PTH-related protein from BEN cells.

Clones of the PTH-related protein were isolated from a cDNA library of BEN cell RNA by screening with oligonucleotide probes based on NH₂-terminal sequence data obtained from the purified protein (13). Two 72-base oligonucleotides were synthesized corresponding to a 24-amino acid NH₂-terminal sequence (8); one used codons based on mammalian frequency tables (14) and the second used codons from PTH for the positions of amino acid match. A total of 250,000 primary, oligo(dT)-primed, cDNA clones in the vector λ gt10 were screened with a mixture of the two

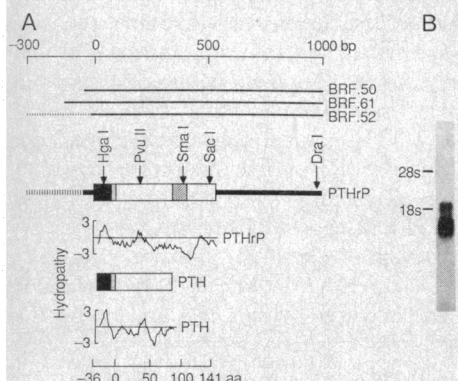
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oligonucleotide probes. Six positive clones were identified with insert lengths from 900 to 1350 bp. The map and DNA sequence of three of these clones (BRF.50, BRF.52, and BRF.61) is shown in Fig. 1. The probes match the cloned DNA sequence either with two 11-bp matches separated by a single mismatch or with two separate 12- and 11-bp matches.

The cloned sequences include a single long open reading frame of 531 bp beginning with a methionine residue (Fig. 1C). No other ATG codons are found in this reading frame. The DNA sequence sur-

Table 1. Amino acid analysis of purified PTH-related protein. A highly purified sample of PTH-related protein from BEN cell medium was chromatographed on a Brownlee C₈ (2.1-mm) column. The peak fraction was used for analysis in a Beckman 6300 amino acid analyzer. The molar yield of each amino acid was converted to residues with the assumption of a total of 140 amino acids (the translated length without tryptophan). ND, not determined.

Amino acid	Residues (sample)	Residues (theoretical)
D+N (Asx)	11.6	12
T (Thr)	9.5	12
S (Ser)	10.5	14
E+Q (Glx)	20.3	17
P (Pro)	9.2	7
G (Gly)	11.9	10
A (Ala)	7.3	5
C (Cys)	ND	0
V (Val)	4.7	5
M (Met)	0	0
I (Ile)	4.6	4
L (Leu)	10.4	12
Y (Tyr)	1.7	2
F (Phe)	2.9	3
H (His)	6.5	8
K (Lys)	19.1	17
R (Arg)	9.9	12
W (Trp)	ND	(1)
Total	140	140



rounding the ATG codon is that expected for the initiation of eukaryotic RNA translation (15). After a 36-amino acid leader, the NH₂-terminal sequence of the mature protein begins. This translated protein sequence matches exactly a 38-amino acid NH₂-terminal sequence determined for the purified protein (with the exception of a single undertermined residue) (Fig. 1C). The amino acid sequence of both tryptic fragments determined from the purified protein is also found in the translated cDNA sequence from amino acids 38 to 50 and 67 to 79. The cDNA clones predict a mature, full-length protein of 141 amino acids with an M_r of 16,000. This compares with an M_r of 18,000 determined by SDS gel electrophoresis for the purified protein (8). The predicted amino acid composition for the 141-amino acid protein also compares well with that determined for the purified material (Table 1). Both the molecular weight and composition data suggest that the purified BEN cell protein contains all or nearly all of the 141 amino acids predicted for the full-length protein. The mature protein contains no cysteine or methionine and no potential NH₂-linked glycosylation sites.

Sequence homology of the translated protein with PTH from several species is shown in Fig. 2. Most of the homology occurs in the first 20 residues although the remainder of the protein does show limited homology with PTH. There is little homology between the leader sequence of the protein and PTH. However, the overall structure of the leader sequence suggests that it may function in a manner analogous to the prepro sequence of

PTH (16). A hydropathy plot (Fig. 1A) shows that the leader sequence contains a hydrophobic core of amino acids flanked by charged residues as expected for a secretion signal sequence (17). Cleavage of this signal sequence after glycine -8 or serine -6 (18) would leave a short, basic pro sequence like that found for PTH which would be cleaved off to give the mature protein.

The COOH-terminal extension of the PTH-related protein beyond the length of PTH starts with a basic peptide of 25 amino acids containing 60% lysine and arginine residues. This is followed by a sequence of 33 amino acids which is not homologous to any known protein. The role of this basic peptide and the sequence that follows will await further studies. However, it is possible that cleavage within the basic sequence could release the COOH-terminal peptide as a newly found hormone. Precursor proteins containing multiple hormones separated by basic sequences have been described in other systems (19, 20).

The DNA sequence of the three clones shown in Fig. 1 is the same in the coding and 3' untranslated regions with the exception of a single nucleotide near the 3' end (legend to Fig. 1). At 23 bp 5' of the initiating ATG, the 5' end of clone BRF.52 diverges from the other two. Presumably, two alternatively spliced RNAs give rise to these two types of cDNA clones that code for the same protein, although a cDNA cloning artifact cannot be excluded. In the 5' sequence of all three clones, only one ATG codon, the initiating ATG, is found in the open reading frame. Figure 1B shows that the RNA from BEN

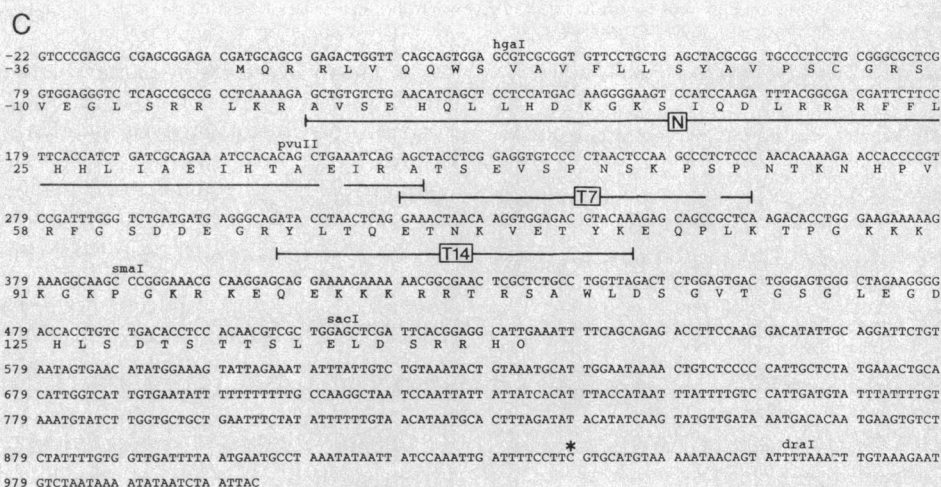


Fig. 1 (A). Map of the cDNA clones and a schematic drawing of the PTH-related protein. The insert of each of the three cDNA clones, BRF.50, BRF.52, and BRF.61, is shown as a single line. The dotted portion of BRF.52 diverges from 50 and 61. The coding region of the protein is shown as a box. Solid box, pre sequence; stippled box, pro sequence; lined box, basic peptide. The hydropathy plots are from the method of Kyte and Doolittle (26). The corresponding coding region of PTH and its hydropathy plot are shown for comparison. **(B)** Blot of PTH-related protein RNA. Polyadenylated RNA from BEN cells was subjected to electrophoresis on a 1% agarose-formaldehyde gel (27), transferred to nitrocellulose, and hybridized to a probe consisting of the full-length cDNA insert of BRF.50. **(C)** DNA sequence of the cDNA clones. The three clones have the same DNA sequence with the exception of bp 938 (*) which is C in BRF.52 and T in BRF.50 and BRF.61, and except for a divergent 5' end of BRF.52. The differing 5' ends are not shown. The underlined amino acids indicate the location of the NH₂-terminal and tryptic amino acid sequences. The gaps in the underlining show residues that were uncertain.

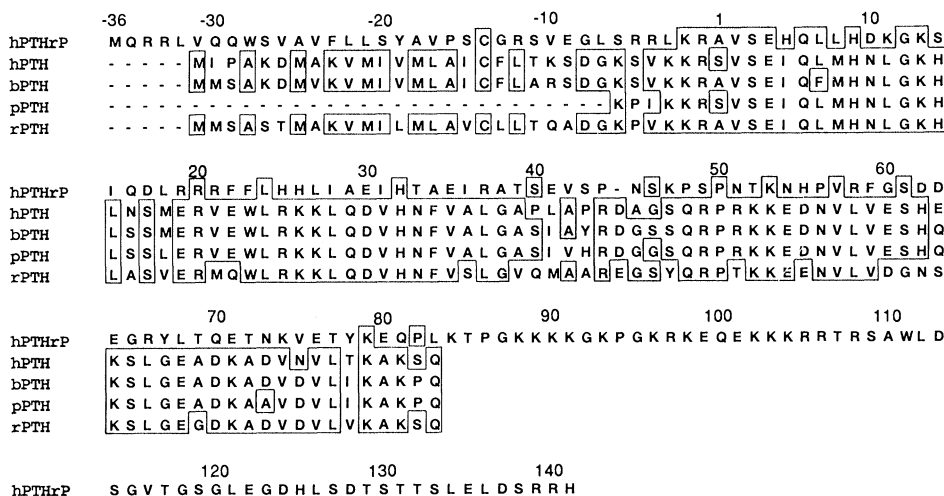


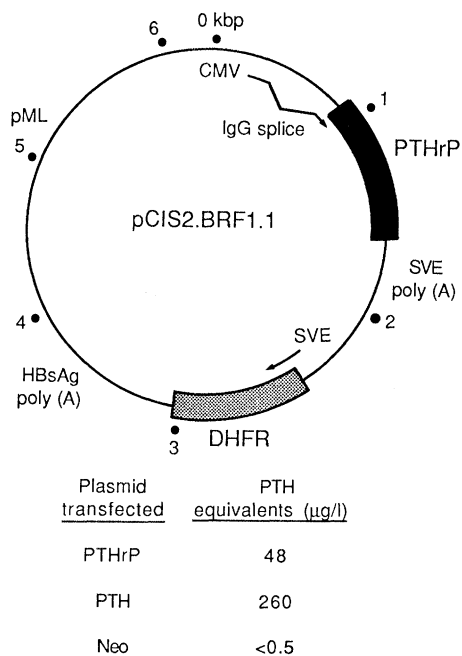
Fig. 2. Homology of PTH-related protein and PTH. The sequences are human PTH-related protein (hPTHrP) reported here; and human (h), bovine (b), porcine (p), and rat (r) PTH (28).

cells coding for the PTH-related protein is a broad band centered at about 1350 bp with some material as high as 1700 bp. Variable splicing in the 5' untranslated region as well as variable 3' poly(A) sequences probably account for this broad band. Clones containing a poly(A) sequence at the 3' end have not yet been isolated. The three clones shown in Fig. 1 all end within 8 bp of one another and contain the poly(A) addition sequence, AA-TAAAA, at bp 983. On the basis of the RNA size estimated from Fig. 1B, it appears that the clones contain nearly all of the 3' untranslated region.

Expression of active PTH-related protein

was achieved by construction of a mammalian expression vector containing the full-length cDNA sequence. Figure 3 shows this vector in which a cytomegalovirus promoter directs the synthesis of the PTH-related protein. This vector was constructed so that the full-length protein would be synthesized, including its putative prepro sequence. We expect that cleavage of this sequence is required for the secretion and generation of an active molecule. A vector was also constructed for the expression of PTH as a positive control (see Fig. 3). When these plasmids were transiently transfected into COS-7 monkey kidney cells,

Fig. 3. (Top) The PTH-related protein expression plasmid pCIS2.BRF1.1. The expression plasmid was constructed by using a vector similar to pF8CIS9080 (29) but with the factor VIII gene from Cla I to Hpa I removed. The complete coding region of BRF.52 was inserted in this vector by using the Dra I site near the 3' end of the cDNA clone. A Cla I site was synthesized and placed 20 bp 5' of the initiating ATG. The human PTH expression plasmid pRK5.PTH1.1 was assembled from a genomic clone containing the COOH-terminal coding region and from synthetic DNA to reconstruct the complete 5' coding portion of the processed gene (30). The expression vector used for PTH is similar to that above, except that it lacks the SV40-dhfr transcription unit (31) in pCIS2.BRF1.1 and has some changes in the spliced 5' untranslated region of the hybrid CMV, immunoglobulin G intron (29). **(Bottom)** Expression of PTH-related protein and PTH in mammalian cells. COS-7 monkey kidney cells (32) were cotransfected with the plasmids described above and pRSVneo (33) (or with pRSVneo alone) by the calcium phosphate precipitation method (34). For 100-mm dishes of cells, 5 μ g of PTHrP or PTH expression plasmid and 1 μ g of pRSVneo were used. After 72 hours, 10- and 100- μ l aliquots of the cell medium were assayed for PTH-like activity by determining the stimulation of cAMP levels in UMR106 cells (8). Cyclic AMP levels were determined by radioimmunoassay (Chemicon, International, San Die-



go). Activity in the COS-7 supernatants is reported as PTH equivalents with a human PTH(1-34) standard curve.

PTH-like activity could be detected in both culture media (Fig. 3, bottom). No activity was found with a control neomycin gene plasmid. PTH-like activity was determined as a stimulation of cAMP levels in the osteoblast-like cell line UMR106 (8).

A PTH-related protein has been isolated and the active protein expressed from recombinant DNA-derived cDNA clones. This demonstrates the existence of a previously unrecognized PTH-like protein as a product of a human lung cancer cell line. The properties of this protein (8) are very similar to those demonstrated in tumor extracts from patients with HHM (9, 10), and it is likely that this protein (perhaps with other related factors) has a role in the pathogenesis of HHM syndrome, acting on kidney to promote calcium retention and phosphorus and cAMP excretion, or on bone to increase resorption, or on both. The identification of this protein provides new insights into the pathogenesis of HHM, and has implications for our understanding of normal calcium and phosphorus metabolism. The protein may be the result of a gene duplication of a common ancestor of the PTH gene, as it has clear homology with PTH itself. The homology of the NH₂-terminal region appears to be sufficient to explain the interaction of the tumor-derived protein with the PTH receptor. The differences in the remainder of the molecule explain why most antisera have failed to detect elevated PTH levels in serum of patients with HHM syndrome (3). The sequence homology can also explain why, with a few antisera used in studies over the past several years, some "PTH" immunoreactivity has been detected, albeit at low levels and nonparallel to standard PTH (21). Furthermore, the application of very high concentrations of PTH antisera has led to the conclusion in some immunohistochemical studies that such tumors do produce PTH (22, 23), even when PTH is not detected in the sera of such patients (23).

The contribution of the PTH-related protein to the biochemical abnormalities in HHM remains to be determined, and indeed the relative contribution of the kidney and the skeleton to hypercalcemia is the subject of debate (3, 4, 24). The high incidence of elevated nephrogenous cAMP in normocalcemic patients with lung cancer (5) suggests that production of the protein may occur even more commonly than suspected. It will be important to investigate the use of sensitive assays for this protein in the early diagnosis and management of lung cancer. Its physiological function could be of considerable interest, particularly in view of its production by human keratinocytes in culture (25).

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13. Polyadenylated RNA was isolated from BEN cells by the LiCl precipitation method [G. Cathala *et al.*, *DNA* **2**, 329 (1983)] and oligo(dT) cellulose chromatography (27). From 5.5 g of BEN cells, 17 mg of total and 700 µg of poly(A)⁺ RNA were isolated. Double-strand cDNA was synthesized with the Amersham system [U. Gubler and B. J. Hoffman, *Gene* **25**, 263 (1983)]. This cDNA was linked, size-selected, and cloned in the λgt10 vector as described [W. I. Wood *et al.*, *Nature (London)* **312**, 330 (1984)]. From 2 µg of BEN poly(A)⁺ RNA, 300 ng of double-strand cDNA was isolated. From 1 ng of this cDNA over 1 million clones were generated. Clones were hybridized to ³²P end-labeled oligonucleotide probes (see text) in 20% formamide, 5× SSC, 50 mM sodium phosphate, pH 7.0, 0.04 g per liter of boiled, sonicated salmon sperm DNA, 0.05% SDS, 5× Denhardt's, and 10% dextran sulfate at 42°C, and washed in 1× SSC containing 0.1% SDS at 42°C. Two 72-base oligonucleotides based on a 24-amino acid NH₂-terminal sequence (8) determined from 20 pmol of pure protein were used: BRF.1, 5'-GCTGTCTCTGAGCATCAGCTGCTG-CATGACAAGGGCAAGTCCATCCAGTCCTTTGAGCGGCGTTCTTCCTG, and BRF.2, 5'-GCTGTGAGTGAACATCAGCTTCTGCATGA-CAGGGCAAATCCATCCAGTCCCTTGAGAG-ACGGTTCCTCTCTG. Sixteen of the amino acid residues have been presented previously (8). In that work a residue was not detected at cycle 17 and there were ambiguities at cycles 18 and 19. For the design of the probes, the sequence of residues 17–19 was assumed to be SFE. A subsequent 38-amino acid NH₂-terminal sequence showed the correct residues to be DLR (see Fig. 1C). Positive clones were subcloned in pUC119, and the DNA sequence determined by the dideoxy chain termination method [F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)]. For the RNA blot, 2 µg of BEN cell poly(A)⁺ RNA were subjected to electrophoresis and hybridized to a primed synthesis labeled (27) BRF.50 Eco RI insert. The hybridization was as above but in 50% formamide, with a wash in 0.2× SSC containing 0.1% SDS at 50°C. Amino acid sequence data were obtained for the PTH-related protein purified as described previously (8) with a final purification by reversed-phase high-performance liquid chromatography (HPLC) (Baker C₁₈ wide bore column). For the NH₂-terminal sequence, approximately 100 pmol of this material was analyzed by sequential Edman degradation with an Applied Biosystems model 470A sequencer [R. E. H. Wettenhall, W. Kudlicki, G. Kramer, B. Hardesty, *J. Biol. Chem.* **261**, 12444 (1986)]. An additional 100 pmol was digested with trypsin (1:10) for 24 hours at 37°C, chromatographed by HPLC, and the sequence of several of the peaks determined. Two of these peaks, T7 and T14, gave usable sequence data.
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Does the Release of Potassium from Astrocyte Endfeet Regulate Cerebral Blood Flow?

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Local increases in neuronal activity within the brain lead to dilation of blood vessels and to increased regional cerebral blood flow. Increases in extracellular potassium concentration are known to dilate cerebral arterioles. Recent studies have suggested that the potassium released by active neurons is transported through astrocytic glial cells and released from their endfeet onto blood vessels. The results of computer simulations of potassium dynamics in the brain indicate that the release of potassium from astrocyte endfeet raises perivascular potassium concentration much more rapidly and to higher levels than does diffusion of potassium through extracellular space, particularly when the site of a potassium increase is some distance from the vessel wall. On the basis of this finding, it is proposed that the release of potassium from astrocyte endfeet plays an important role in regulating regional cerebral blood flow in response to changes in neuronal activity.

ROY AND SHERRINGTON, IN 1890, suggested that "the brain possesses an intrinsic mechanism by which its vascular supply can be varied locally in correspondence with local variations of functional activity" (1, p. 105). Such an intrinsic homeostatic mechanism would help to maintain an adequate supply of oxygen and nutrients to the brain despite widely varying levels of neuronal activity. Although the existence of such a regulatory process has been established (2–5), the mechanism that links neuronal activity and regional cerebral blood flow (rCBF) remains unknown. Interstitial concentrations of potassium and hydrogen ions, adenosine, and several neurotransmitters vary with neuronal activity. These substances all cause changes in arteriole diameter (6, 7) and have been implicated in the regulation of rCBF. However, the relative importance of each of these factors is not known.

Extracellular K⁺ concentration ([K⁺]_o) varies widely during periods of neuronal activity and can rise from a quiescent level of approximately 3 mM to a maximum level of more than 10 mM (8). Cerebral arteries and

arterioles (but not capillaries) are extremely sensitive to changes in K⁺ concentration, increasing in diameter as much as 50% in response to a change in [K⁺]_o from 3 to 10 mM (9, 10). This sensitivity to K⁺ could be an important factor in regulating rCBF. Potassium released by active neurons could diffuse through extracellular space to the abluminal wall of arterioles and cause arteriole dilation. The resulting decrease in vascular resistance (11) would increase rCBF, thus bringing a greater supply of oxygen to precisely the region where it is needed, to the activated portion of the brain. However, arterioles are widely spaced within the brain [they are often separated by more than 500 µm (12)] and may not necessarily be near regions of activated tissue. Thus, the K⁺ released by active neurons would have to diffuse tens or hundreds of micrometers before reaching arterioles and effecting dilation.

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