An Enzyme-Modified Microelectrode That Detects Choline Injected Locally into Brain Tissue

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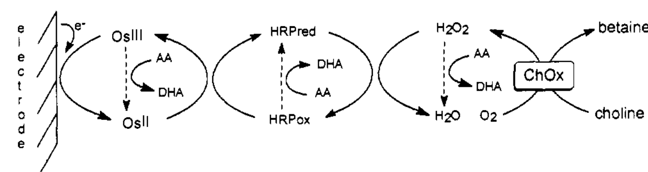
The development of microelectroanalytical techniques for monitoring neurotransmitter levels in the brain extracellular fluid of living animals has been both challenging and fruitful. Much effort has been focused on the catecholamines dopamine[1a,b] and norepinephrine,[1c,d] which are particularly amenable to electrochemical detection. Motivated in part by the successes of Adams and others, several groups are developing microsensors for the in vivo detection of other classes of neurotransmitters.[2] We report herein that an amperometric sensor based on a microelectrode modified with an enzyme-containing redox polymer film permits the detection of choline, the precursor and metabolite of acetylcholine, injected locally into brain tissue. This is significant not only because of the biochemical and potential pharmaceutical importance of choline but also because the microelectrode technique described below is a forerunner to the in vivo detection of acetylcholine itself. Furthermore, as far as we are aware, this is the first time that a redox-polymer-based sensor has been demonstrated to function in vivo.

Recently, we reported that a prototype choline sensor[2c] could be constructed by using a cross-linkable redox polymer[3] to immobilize choline oxidase (ChOx) and horseradish peroxidase (HRP) onto conventional glassy carbon electrodes. Scheme I shows the sequence of events by which an electrochemical signal is generated for choline, which itself is not electrochemically active. The enzymatic oxidation of choline leads to the formation of H_2O_2, which is in turn reduced by HRP. The redox polymer, in addition to immobilizing the enzymes, mediates electron transfer between HRP and the electrode, thereby allowing the detection of choline at an applied potential slightly negative of SCE. This is extremely beneficial as few of the many electrochemically active compounds in brain extracellular fluid are oxidized or reduced in this potential region. A major consideration in the development of in vivo electroanalytical methods for neurotransmitters is ascorbate (AA), which is a potent reducing agent present at rather high levels in brain extracellular fluid (200-400 μM).[4] Even though the choline sensor is operated at a potential where little electrooxidation of ascorbate occurs, ascorbate can still interfere with choline detection by the reactions indicated in Scheme I with dashed lines. The oxidation of ascorbate to dehydroascorbate (DHA) is chemically irreversible, so there is no opportunity to recover any redox equivalents consumed by these interfering reactions. It is imperative, therefore, that ascorbate be rejected from the enzyme-containing layer if choline is to be detected in ascorbate-containing fluids such as brain extracellular fluid.

Scheme I



Nafion is a permselective material which has been widely used to diminish the interference of ascorbate with electrochemical sensors,[5a,b] including enzyme-based sensors.[5c,d] Next, both in vitro and in vivo results obtained with Nafion-coated choline microsensors are presented.

Carbon fiber cylinder electrodes (Thornel P55, 5-μm radius by 200-300-μm length) were prepared by usual methods[6] and manually coated with an aliquot of pH 7.4 phosphate buffer containing 0.37 mg/mL ChOx, HRP, and redox polymer and 0.22 mg/mL cross-linker. After being cured under ambient conditions for 24-48 h, the modified microelectrodes were soaked in ultrapure water for 15 min and dried again for 1-2 h. Finally, the microelectrodes were dip-coated with Nafion (Aldrich) and dried again under ambient conditions for 1-2 h before use. For in vitro calibration, the microelectrodes were mounted in the outlet of a flow system with a loop injector and poised at a potential of -0.1 V versus a SCE. Output of the current amplifier (Keithley 427) was monitored with a PC via an analog-to-digital converter.

Figure 1 compares the current response recorded during separate 25-s injections of 50 μM choline into the flow system in the absence and presence of 200 μM ascorbate. The data show that choline can be detected in the presence of ascorbate with these Nafion-coated microsensors. In comparison, without Nafion (data not shown), interference by ascorbate is so severe that detection of choline is prevented altogether. In the presence of 200 μM ascorbate, the detection limit for choline is 10 μM, which is a physiologically relevant choline concentration. The Nafion-coated microsensors respond rapidly (within ca. 2 s) to step changes in the choline concentration, although the signal continues to drift after the initial rapid response. This temporal response is attributed to the uneven thickness of the manually prepared polymer layers, which can be observed by SEM. Thus, in the long term, elimination of the remaining effect of ascorbate on the choline sensitivity and further improvement of the temporal response of the sensors is called for. However, the sensors we have prepared so far are completely adequate for the aim of this Communication, which is to show that these choline microsensors are suitable for in vivo measurements.

For initial in vivo tests of the microsensors, rats were anesthetized and placed in a stereotaxic surgical frame equipped with micromanipulators that permit small electrodes to be implanted into brain tissue. According to the experimental design of Adams,[7a] a choline microsensor and a micropipet filled with a 100 mM choline solution were mounted side-by-side with their tips separated by approximately 1 mm. The micropipet was connected by poly(tetrafluoroethylene) tubing to a 50-μL gastight syringe mounted into a syringe driver (Sutter Instruments, Model NA-1). The assembly was lowered into the striatum of the rat

(1) (a) Kawagoe, K. T.; Garris, P. A.; Wiedemann, D. J.; Wightman, R. M. *Neuroscience* **1992,** *51,* 55-64. (b) Kennedy, R. T.; Jones, S. R.; Wightman, R. M. *Neuroscience* **1992,** *47,* 603-612. (c) Ghasemzadeh, M. B.; Capella, P.; Mitchell, K.; Adams, R. N. *J. Neurochem.* **1993,** *60,* 442-448. (d) Renner, K. J.; Pazos, L.; Adams, R. N. *Brain Res.* **1992,** *577,* 49-56.

(2) (a) Pantano, P.; Kuhr, W. G. *Anal. Chem.* **1993,** *65,* 623-630. (b) Kuhr, W. G.; Barrett, V. L.; Gagnon, M. R.; Hopper, P.; Pantano, P. *Anal. Chem.* **1993,** *65,* 617-622. (c) Garguilo, M. G.; Huynh, H.; Proctor, A.; Michael, A. C. *Anal. Chem.* **1993,** *65,* 523-528. (d) Kar, S.; Arnold, M. A. *Anal. Chem.* **1992,** *64,* 2438-2443. (e) Kawagoe, J. L.; Niehaus, D. E.; Wightman, R. M. *Anal. Chem.* **1991,** *65,* 2961-2965.

(3) Gregg, B. A.; Heller, A. *J. Phys. Chem.* **1991,** *95,* 5970-5975.

(4) Schenk, J. O.; Miller, E.; Gaddis, R.; Adams, R. N. *Brain Res.* **1982,** *253,* 353-356.

(5) (a) Brazell, M. P.; Kasser, R. J.; Renner, K. J.; Feng, J.; Moghaddam, B.; Adams, R. N. *J. Neurosci. Methods* **1987,** *22,* 167-172. (b) Kristensen, E. W.; Kuhr, W. G.; Wightman, R. M. *Anal. Chem.* **1987,** *59,* 1752-1757. (c) Navera, E. N.; Suzuki, M.; Tamiya, E.; Takeuchi, T.; Karube, I. *Electroanalysis* **1993,** *5,* 17-22. (d) Harrison, D. J.; Turner, R. F. B.; Baltes, H. P. *Anal. Chem.* **1988,** *60,* 2002-2007.

(6) Michael, A. C.; Justice, J. B., Jr. *Anal. Chem.* **1987,** *59,* 405-410.

(7) (a) Rice, M. E.; Gerhardt, G. A.; Hierl, P. M.; Nagy, G.; Adams, R. N. *Neuroscience* **1985,** *15,* 891-902. (b) This calculation of *D* assumes a point source of diffusing material. The Appendix in ref 7a provides a correction factor that takes into account the volume of the injected solution. We have not applied this correction, which is minor for our experimental conditions (ca. 20%), so the reported value of *D* may be regarded as an upper limit.

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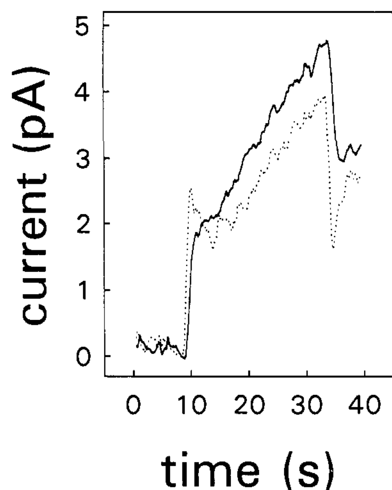

Figure 1. Current response of a Nafion-coated choline microsensor in the flow-injection analysis system. Signals were recorded during the 25-s injection of 50 μM choline with (dotted line) and without 200 μM ascorbate (solid line). The sensor was operated at a constant potential of −0.1 V vs SCE.

brain, and the response of the sensor was monitored following the ejection of 375 nL of choline solution from the micropipet.

Figure 2 shows the response of the microsensor to the local injection of choline into brain tissue. Control data were obtained in order to confirm that the response is due to the injected choline rather than artifact. First, no response to a choline injection could be obtained with a microsensor lacking ChOx. Second, no response could be obtained with a choline microsensor when a choline-free solution was injected into brain tissue. The time course of the response shown in Figure 2 depends primarily on diffusion since the saturable uptake of choline by striatal tissue has a K_m of 1–5 μM,[8] far below the injected concentration. Adams has shown that t_{max}, the time required for the response to reach its maximum value, is related to D, the diffusion coefficient of the injected material, and d, the spacing between the sensor and the pipet, by the expression $D = d^2/6t_{max}$.[7b] The diffusion coefficient of choline determined with this expression is $(3.3 \pm 0.1) \times 10^{-6}$ cm²/s ($\bar{x} \pm$ SD, eight observations from three animals), which is in excellent agreement with values reported by Adams[7] for a number of small molecules in rat striatum, confirming that

(8) Cooper, J. R.; Bloom, F. E.; Roth, R. H. *The Biochemical Basis of Neuropharmacology*, 4th ed.; Oxford University Press: New York, 1982; p 80.

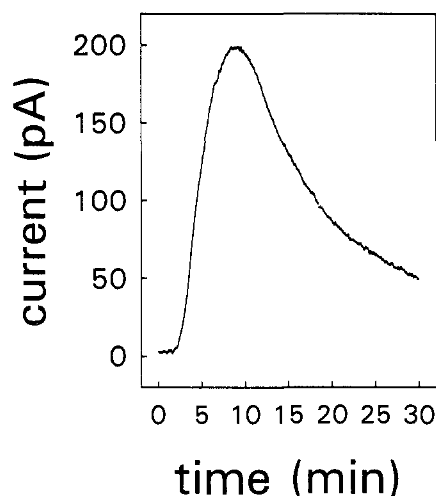

Figure 2. Current response of the microsensor following the local injection of 100 mM choline into brain tissue. The sensor was held at an applied potential of −0.1 V vs Ag/AgCl.

the microsensors are able to provide temporal information about choline levels in brain extracellular fluid.

After the microsensors were removed from the rat brain, they were recalibrated in vitro. Exposure to brain tissue caused less than a 20% change in the choline sensitivity of the sensors. This can be attributed to the biocompatibility of the Nafion overlayer, which has been noted before.[5d] Using the in vitro calibration data, the measured current can be converted to choline concentration. When this is done, the maximum concentration (ca. 1 mM) observed following each injection is found to agree, within a factor of 2, with that predicted by diffusion equations (ca. 2 mM).[7] This provides preliminary evidence that the microsensors also provide quantitative information about choline levels in brain extracellular fluid. Despite the excellent agreement between the calibrated and predicted concentrations, the extent to which the availability of dioxygen in brain extracellular fluid (see Scheme I) affects the in vivo performance of these microsensors remains to be seen. Nevertheless, the data presented demonstrate that dynamic changes in choline can be monitored in the brain extracellular fluid of the living rat with minimal interference from high levels of ascorbate.

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