Increased Intracellular Calcium in Rat Anterior Piriform Cortex in Response to Threonine After Threonine Deprivation

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Magrum, Linda J., M. Anne Hickman, and Dorothy W. Gietzen. Increased intracellular calcium in rat anterior piriform cortex in response to threonine after threonine deprivation. J. Neurophysiol. 81: 1147–1149, 1999. The anterior piriform cortex (APC) may serve as the chemosensor for amino acid (AA) deficiency in rats. To investigate the mechanism by which the APC recognizes a limiting indispensable AA (IAA), we examined changes in intracellular calcium ([Ca\(^{2+}\)]\textsubscript{i}) in APC slices after culture in medium with or without threonine (Thr) or lysine (Lys). The addition of 1 or 10 mM Thr to slices previously incubated in Thr-devoid medium resulted in a significant and sustained increase in [Ca\(^{2+}\)]\textsubscript{i}, compared to control slices; an effect not seen when isoleucine, another IAA, was added. Similar results were seen when lysine, but not threonine, was added to slices incubated in lysine-devoid medium. The rise in [Ca\(^{2+}\)]\textsubscript{i}, resulting from the addition of the limiting IAA to deficient slices may be linked to enhanced activity of the appropriate AA transporter. This is suggested by preliminary findings that serine, a small neutral AA that uses the same transporter as threonine, gave rise to an enhanced response in the Thr-deficient slice.

METHODS

Dams and litters of Sprague-Dawley rats were housed in polyethylene cages at 22 ± 2°C (SE) on a 12:12 light:dark cycle. Dams were given access ad libitum to rat chow (5012, PMI Feeds) and water. Protocols followed NIH guidelines as approved by the U. C. Davis Animal Use and Care Committee. Rat pups, aged 7 to 21 days, were killed by decapitation. A portion of the brain containing the APC was prepared by making a transverse cut at the level of the optic chiasm; then with the use of the cut surface of the anterior portion of the brain as a base, a horizontal cut was made at the level of the olfactory bulb. Tissue was glued (cyanoacrylate) to the micromtome stage and submerged in a bath of Earle’s Balanced Salt Solution (EBSS, Sigma) maintained at 0–4°C and equilibrated with 95% O\(_2\)-5% CO\(_2\). Slices 150–200 μm thick were made using a Campden Instruments 752 M Vibroslice. Slices were 18° from a true transverse section (i.e., perpendicular to the pial surface) to preserve the integrity of the apical dendrites of the pyramidal cells in layer II. Ten to 15 slices, rostral to the closure of the anterior commissure, were prepared and transferred to oxygenated EBSS. Six to 8 of the best slices were selected from each rat, and whenever possible, one hemisphere was transferred to complete medium (control), the other to medium devoid of either threonine (−Thr) or lysine (−Lys). The medium was prepared according to Brewer et al. (1993) with amino acids (injectable medical grade) from Ajinomoto and all other components (puriss. grade) from Fluka Chemika-Biochemika. Slices were transferred to 30 mm Millicell-CM culture plate inserts (0.4 μm, Millipore), then placed over 1 ml of the appropriate medium in six-well polystyrene culture plates and incubated for a period of 4–6 h at 37°C in a 5% CO\(_2\) atmosphere. Slices were washed from inserts with EBSS, placed in 2 ml EBSS containing 5 μM fura-2 acetoxyethyl ester (fura-2 AM, Molecular Probes) and incubated for an additional 45 min. Slices were rinsed three times and maintained in EBSS bubbled with 95% O\(_2\)-5% CO\(_2\). Slices were transferred to a glass coverslip held in a clamp chamber (Medical Systems, Chicago, IL) and were restrained with a device made from a ring of propylene, covered on one side with a grid of silk suture thread. Each slice was covered with an initial volume of 360 μl EBSS. This apparatus was placed on an inverted microscope (Nikon Diaphot) equipped with a temperature-controlled stage maintained at 37°C. The APC was located under ×10 magnification and layer II, the pyramidal cell body layer, was viewed under ×20 magnification. Fura-2 was excited alternately at 340 and 380 nm; fluorescence emission was recorded at 510 nm. Ratio imaging was carried out using an ImageMaster Ratio Fluorescence Imaging System consisting of a Photon Technology International Deltascan 4000 dual wavelength monochromator, a Hamamatsu intensified CCD camera, and ImageMaster software. Experiments typically examined three to eight each control and deficient slices from one rat. For experiments with threonine as the limiting IAA, a total of 62 control and 70 deficient slices were imaged from a total of 28 rats. Confirmation of these results with lysine was carried out by imaging 20 slices from a total of 5 rats, with approximately equal numbers of control and deficient slices. Results are expressed as the ratio of fluorescence emission obtained at excitation wavelengths of 340 and 380 nm (R\(_{340/380}\)). R\(_{340/380}\) was chosen for expressing results because regions of interest (ROIs) imaged within any given slice showed considerable variation in initial
Ca\(^{2+}\) level, but similar magnitude of change in [Ca\(^{2+}\)]\(_i\) when expressed as the ratio of fluorescence emission.

**RESULTS**

APC slices incubated with either complete, −Thr, or −Lys medium were imaged during the introduction of a number of amino acids (AAs), all at a final concentration of either 1 or 10 mM. In −Thr and control slices the effects of addition of threonine, isoleucine, serine, alanine, and two other AAs with known neurotransmitter activity, glycine and glutamic acid, were examined. In −Lys and control slices the effects of addition of lysine, threonine, and glutamic acid were examined. The addition of AAs was followed by 50 mM KCl.

In about 20% of the brains examined, there was a clearly distinguishable rise in [Ca\(^{2+}\)]\(_i\) when the limiting IAA was added to a deficient slice. This effect was never seen in control slices. In some brains, more than one deficient slice responded to the limiting IAA and in others, one or none. The response was seen in brain slices from rats across the entire age range of 7 to 21 days. Figure 1 shows the response to threonine in a −Thr slice (Fig. 1A) and its control from the same rat (Fig. 1B). In Fig. 2, using only one responding deficient slice and its control per rat, results of threonine addition were expressed as the change in R\(_{340/380}\) in relation to baseline before AA addition (Fig. 2A) and in relation to the response to glutamic acid (Fig. 2B). By either measure, the addition of threonine to −Thr slices gave rise to a significant increase (Student’s t-test, \(n = 5\), \(P = 0.002\), and \(P = 0.01\), respectively) in R\(_{340/380}\) and thus in [Ca\(^{2+}\)]\(_i\). That this effect is not unique to threonine as a limiting IAA is shown by Fig. 3 in which an increase in [Ca\(^{2+}\)]\(_i\) is seen in response to lysine addition to a −Lys slice (Fig. 3A) but not its control (Fig. 3B). This effect was found in slices of two of five brains examined and was significant (Student’s t-test, \(n = 2\), \(P = 0.009\)) when expressed as a percentage of the response to glutamic acid.

To determine whether the response in −Thr slices was unique to the limiting IAA, we conducted experiments in which another IAA, isoleucine, was added first, followed by threonine. In the responsive slices (3 of 8 brains examined) the addition of threonine to −Thr but not control slices resulted in increased [Ca\(^{2+}\)]\(_i\), whereas the addition of isoleucine failed to do so in either control or −Thr slices [analysis of variance (ANOVA); F\(_{3,8}\) = 15.766, \(P = 0.04\)].

We also examined the effect of other small neutral amino acids on [Ca\(^{2+}\)]\(_i\) in −Thr slices. These AAs are presumably transported into brain cells by the same transport protein as is threonine (Shotwell et al. 1983). Serine addition to both −Thr and control slices caused an increase in [Ca\(^{2+}\)]\(_i\), with the response being more pronounced in deficient slices of two of five rats examined. In addition, serine elicited an increase in [Ca\(^{2+}\)]\(_i\) in −Thr, but not control slices, after [Ca\(^{2+}\)]\(_i\) was elevated by prior addition of both threonine and glutamic acid.

**DISCUSSION**

The increase in [Ca\(^{2+}\)]\(_i\) in response to threonine in −Thr slices and to lysine in −Lys slices suggests that Ca\(^{2+}\) signaling in APC pyramidal cells may be an early, if not the first, mechanism by which repletion of a limiting IAA is recognized.
in the brain. The fact that this effect was not seen in every deficient slice, nor in every ROI imaged, may be explained by the hypothesis that there is a specific region within the APC that functions as the chemosensor. A precedent for this hypothesis comes from the study of the APC in seizures, in which it was proposed that a specific region, the area tempestas, is highly seizurogenic in response to chemical stimulation (Gale 1989). Because areas of the brain other than the APC were not examined, it is not possible to conclude that the increase in [Ca2+]i, in response to a limiting IAA, is unique to the APC.

Neither the addition of the nonlimiting IAA isoleucine to −Thr slices, nor the addition of the nonlimiting IAA threonine to −Lys slices, led to an increase in [Ca2+]i, in either control or deficient slices. These results suggest that this effect is specific to the limiting IAA; however, they do not rule out the possible involvement of specific AA transporters.

Results of preliminary experiments support the hypothesis that the mechanism by which threonine exerts its effect on [Ca2+]i, in −Thr slices, may involve a transporter of small neutral AAAs. Serine addition led to increased [Ca2+]i, in both −Thr and control slices, suggesting that it may have neurotransmitter-like activity in our preparation (Smith 1996); however, the response to serine in −Thr slices appeared greater than in control slices and was observed even after the elevation of [Ca2+]i, by glutamic acid.

These results support the hypothesis that the APC functions in recognition of a limiting IAA. An increase in [Ca2+]i, may be the first signal by which this recognition is accomplished, leading to a cascade of events which ultimately influences the behavior of the animal to accept or reject a food on the basis of its effect on AA homeostasis.

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