Calcium dependence of rat brain tryptophan hydroxylase

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Summary. The effects of various concentrations of ionized Ca were examined on the activity of rat brain tryptophan hydroxylase previously treated with EGTA. Within the range of ionized Ca-concentrations thought to be physiologically important (10^{-8} to 10^{-5} M), no significant activation of the enzyme occurred, although activation was observed at higher concentrations of the metal.

A number of mechanism have been proposed for the regulation of tryptophan hydroxylase which is the rate-limiting enzyme in the biosynthesis of serotonin, including product inhibition[1], substrate availability[2] and activation by Ca[3,4]. These later effects of Ca may be physiologically important since it is known that the level of intracellular free Ca fluctuates with the state of excitability of the cell and that Ca enters upon depolarization to produce a transient increase in free Ca[5] which might possibly then activate various enzymes such as tryptophan and tyrosine hydroxylases[3,4,6]. Little is known of the level of free Ca within resting and activated neurons of the vertebrate CNS, but studies with the giant squid axon[7] have shown that the free Ca-concentration in the resting axon is about 2×10^{-8} M and that this increases to approximately 3×10^{-6} M Ca^{++} upon depolarization[8]; it may also be noted that a generally similar range of free Ca-concentrations is found between the resting and stimulated states of other excitable tissues such as skeletal muscle[9] and that inhibition of bovine brain microtubule polymerization has been demonstrated[10] at a concentration of 3×10^{-6} M ionized Ca. Thus, if these free Ca-levels in the squid axon approximate those of neurons in the vertebrate CNS, it would appear that the physiologically important range of free Ca-concentrations is from 2×10^{-8} M to around 3×10^{-6} M. However, the studies[3,4] on the in vitro activation of the rat brain hydroxylase by Ca have shown that a Ca-concentration of 5×10^{-4} M is required to produce statistically significant activation of this enzyme, and that 10^{-3} M Ca produces decreased K_m for both substrate and pterin cofactor without altering V_{max}. From the above considerations it would thus appear that these latter studies[3,4] employed supraphysiological Ca-concentrations, and it was therefore of interest to determine if the rat brain enzyme is activated by Ca-ions at concentrations which might be expected to occur intracellularly within the CNS.

Methods. Tryptophan hydroxylase activity was determined in a high speed supernate of rat midbrains, prepared and assayed fluorimetrically as described by Knapp et al.[3], but with the incubation time at 37 °C decreased to 15 min, and with the concentrations of tryptophan and 6 MPH_4 (6-methyl, 5,6,7,8-tetrahydropterine) being sub-

1 M. Hamon, S. Bourgoin and J. Glowinski, J. Neurochem. *20*, 1727 (1973).
2 A. Tagliamonte, P. Tagliamonte, J. Perez-Cruet, S. Stern and G. L. Gessa, J. Pharmac. exp. Ther. *177*, 475 (1971)
3 S. Knapp, A. J. Mandell and W. P. Bullard, Life Sci. *16*, 1583 (1975).
4 M. Boadle-Biber, Biochem. Pharmac. *24*, 1455 (1975).
5 P. F. Baker, A. L. Hodgkin and E. B. Ridgeway, J. Physiol. *218*, 7091 (1971).
6 V. H. Morgenroth, M. Boadle-Biber and R. H. Roth, Mol. Pharmac. *11*, 427 (1976).
7 J. Requena, R. Dipolo, L. J. Mullins, F. J. Brinley and A. Scarpa, Fed. Proc. *35*, 846 (1976).
8 P. F. Baker, Prog. Biophys. Mol. Biol. *24*, 177 (1972).
9 W. Hasselbach, Prog. Biophys. Mol. Biol. *14*, 167 (1964).
10 A. C. Rosenfeld, R. V. Zackroff and R. C. Weisenberg, FEBS Lett. *65*, 144 (1976).

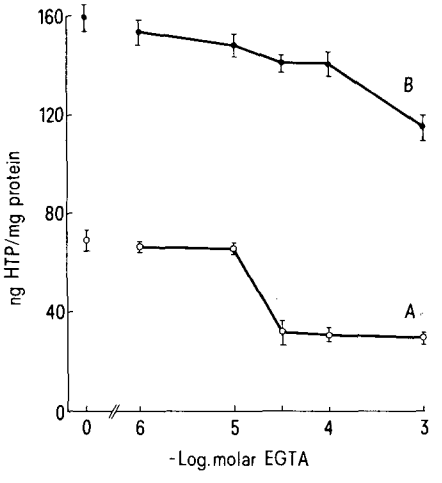

Fig. 1. Effect of EGTA on tryptophan hydroxylase activity. Curve A: Brain supernate was pre-incubated for 5 min with various concentrations of EGTA, with no added Ca; reaction started by addition of 6 MPH_4. Curve B: supernate pre-incubated with EGTA, then Ca added to provide 2×10^{-3} M Ca in excess of respective EGTA concentration, then 6 MPH_4 added. Data represents means $\pm$ SE for 4 preparations.

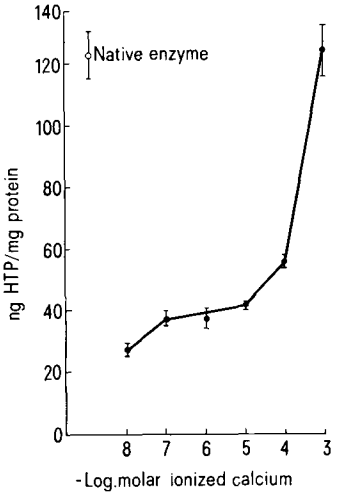

Fig. 2. Effect of ionized Ca-concentration on tryptophan hydroxylase activity. Brain supernate was pre-incubated for 5 min with 10^{-4} M EGTA, then Ca added to provide ionized Ca-levels calculated from data of Weber and Winecur, or added in excess of EGTA (10^{-4} and 10^{-3} Ca^{++}-points). Data from 4 preparations. Activity at 10^{-8} M Ca^{++} was compared with activities at other Ca^{++}-levels, using Student's t-test and taking < 0.05 as being statistically significant.

saturating at 0.2 mM and 0.05 mM respectively. Approximately 2 mg of protein was used, in a final incubation volume of 0.5 ml.

Results and discussion. In the first experiment, EGTA (ethylene glycoltetraacetic acid) at various concentrations was preincubated at 37 °C for 5 min with the brain supernate to complex endogenous Ca^{++} which might normally be associated with the hydroxylase or which might have been liberated within the homogenate after redistribution from various compartments such as mitochondria; the reaction was then initiated by addition of 6 MPH_4. Curve A of figure 1 shows that EGTA has little effect below 5×10^{-5} M, but at this concentration enzymatic activity is markedly suppressed and then remains constant with increasing EGTA concentrations. The possibility that EGTA had deleterious effects in addition to complexing the Ca was tested by pre-incubating with EGTA, then adding an excess of Ca to give a final concentration of 2×10^{-3} M free Ca in all cases. Curve B shows that the reactivation of the enzyme by Ca varies after an initial exposure to EGTA, depending on the EGTA concentration. Reactivation by Ca is constant after exposure of up to 10^{-4} M EGTA, but is suboptimal after exposure to 10^{-3} M EGTA, suggesting that this higher concentration of chelating agent damages the enzymes. The effect of various concentrations of ionized Ca on enzymatic activity were then studied, by pre-incubating the brain extract with 10^{-4} M EGTA, followed by addition of Ca to yield the indicated ionized Ca-concentrations, as calculated by the method of Weber and Winecur[11]. Figure 2 shows that when the ionized Ca is adjusted to 10^{-8} M there is a reduction in activity from 70 ng of 5-hydroxytryptophan/mg protein seen with the native enzyme to 27 ng/mg at this low Ca^{++}-concentration. Increasing the free Ca-levels up to 10^{-5} M shows a trend of increased enzymatic activity which however does not become statistically significant until the Ca^{++}-concentration reaches 10^{-4} M. A sharp break in the curve is then seen, with marked increase in activity at 10^{-3} M free Ca, as observed by other workers[3,4]. The finding that the activity of the native enzyme is higher than that seen in presence of 10^{-4} Ca^{++} may merely reflect an activation of the enzyme by Ca liberated upon homogenization from mitochondria or other Ca-compartments, and as such would have little physiological significance.

The effects of other divalent metals were also studied in experiments (data not shown) in which brain supernate was pre-incubated with 10^{-4} EGTA, followed by addition of 2×10^{-4} M metal to yield approximately 10^{-4} free metal; hydroxylase activity was then assayed. Barium, zinc, strontium, magnesium and manganese had no effect on the EGTA-treated, Ca-depleted enzyme, but Fe^{++} was considerably more active than Ca. It has been suggested[12] that activation of tryptophan hydroxylase by Fe in vitro is due to the breakdown of H_2O_2 produced by the non-enzymatic oxidation of the reduced pterin cofactor; however, Fe could not be replaced by catalase in our experiments.

These experiments thus demonstrate that rat brain trytophan hydroxylase is not significantly activated by ionized Ca within the concentration range predicted from giant squid axon studies to be physiologically important, although activation becomes statistically significant at 10^{-4} M Ca^{++} and is very evident at higher metal concentrations, as shown by other workers[3,4]. It would thus seem that a direct activation of the hydroxylase by Ca^{++} has little physiological significance, and that other mechanisms must be sought to explain the regulation of this enzyme in vivo.

11 A. Weber and S. Winecur, J. biol. Chem. *236*, 3198 (1961).
12 P. A. Friedman, A. H. Kappelman and S. Kaufman, J. biol. Chem. *247*, 4165 (1972).

Transcriptional effects of 5-bromo-2′-deoxyuridine in post-implantation mouse embryos

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Summary. Administration of 5-bromo-2′-deoxyuridine to pregnant mice on the 6th day p.c. brings about significant reduction in the incorporation of ^{3}H-uridine into RNA of 11-day-old embryos and causes elimination of a RNA species characteristic of that developmental stage.

There is ample evidence to show that 5-bromo-2′-deoxyuridine (BUdR) inhibits differentiated functions of a variety of eukaryotic cells[3–6]. This effect has been purported to be mediated through the suppression of transcription of specific genes subsequent to the incorporation of the analogue into DNA[5–9]. Alternatively, it has been suggested that normal regulatory processes might be disrupted by thymine substitution of the target DNA by BUdR[8,10]. The former possibility is born out by the observations that the suppressive effects of BUdR on gene function are reversible by subjecting the substituted DNA to the dilution effects of growing the cells in BUdR-free medium[11–14]. The role of the latter course of events in determining the cellular responses to BUdR could be ascertained by studying the influence of the analogue exposure on the transcriptional process in a system, like developing embryos, in which interdependent sequential gene action is established. The present experiment has been designed on the basis of this concept.

Materials and methods. BALB/c$^+$ female mice $2^1/_2$–3 months of age, were mated with adult male mice of proved fertility and mating was confirmed by the observation of vaginal plug. On the 6th day postcoitum each pregnant mouse was given 2 injections of 500 µg of BUdR (Calbiochem.) dissolved in physiological saline via i.v. route. The interval between the injections was 7 h. On the 11th day of pregnancy, the animals were administered ^{3}H-uridine (C.E.N./S.C.K. Mol, Belgium; specific activity: 10 Ci/mmole) at 3.5 µCi/g body weight. 2 h after the injection, the mice were sacrificed and embryos collected. After washing in chilled saline, the embryos were pooled and used for RNA extraction by phenol-SDS method essentially as described by Brown and Littna[15].

Radioactivity of the RNA was determined by adding Instagel (Packard Inc.) to a known volume of the preparation and counting in a Packard liquid scintillation system. Counting time was so selected as to attain a