SYNAPTIC PLASMA MEMBRANE PROTEIN SYNTHESIS : SELECTIVE INHIBITION BY CHLORAMPHENICOL IN VIVO.

Galo Ramirez [1]

Institute of Neurobiology,Faculty of Medicine,University of Göteborg,Sweden.

Received November 27,1972

SUMMARY.- Chloramphenicol produces a selective and strong additional inhibition of the amino acid incorporation into synaptic plasma membrane proteins when the general cell protein synthesis,in the rat brain,is inhibited by adequate doses of cycloheximide.The chloramphenicol inhibitory effect is also noticeable in both cell body and nerve ending mitochondria but it appears to be more intense in the synaptic plasma membrane.It is then concluded that, in confirmation of our previous findings in vitro,there exists in the synaptic junction of the mammalian brain neurons an autonomous protein-synthesizing system highly sensitive to chloramphenicol.

The possibility of a local regulation of the synaptic events makes extremely important the existence of a certain degree of metabolic autonomy in the nerve ending structures.Recently we have shown that,in addition to nerve ending mitochondria,rat brain synaptosomal membranes can incorporate amino acids into protein in vitro in a chloramphenicol-sensitive and cycloheximide-resistant manner.This unusual protein-synthesizing system is localized in that portion of the nerve ending membrane which constitutes the real synaptic contact,the so-called junctional complex (1,2).

Since this phenomenon could be in some way related to the artificial conditions occurring in in vitro experiments,and might not have a functional counterpart in the living animal,it appeared necessary to try to confirm it by using the in vivo approach.In the present paper I describe the results of two experiments that demonstrate the additional inhibitory effect of chloramphenicol on protein synthesis in different subcellular fractions of rat brain when the animal is already under "maximal" protein

---

[1] Permanent address: Department of Molecular Biology,Centro de Investigaciones Biológicas,Velázquez 144, Madrid-6, Spain.

synthesis inhibition by cycloheximide.

Those results fully confirm our previous results in vitro ,and add new interesting features to our present knowledge of the inhibitory effects of chloramphenicol on mammalian cells (3).

EXPERIMENTAL

Chloramphenicol succinate was purchased from Erco Läkemedel AB,Sweden,and cycloheximide from Sigma Chemical Co.L-(4,5-^{3}H) leucine (46.9 Ci/mmole) and L-(U-^{14}C) leucine (342 mCi/mmole) were obtained from The Radiochemical Centre,Amersham,England.All other chemicals were reagent grade.

Four young male,Sprague-Dawley rats,weighing around 200 g each,were used in each experiment.For purposes of description these rats can be further divided in two groups,A and B,each one comprising two rats.All four rats received a subcutaneous dose of cycloheximide of 120 mg/kg of body weight (4), and rats B received simultaneously a chloramphenicol saturation dose of 600 mg/kg of body weight (5),by the same route.Fifteen minutes later,rats A were given a bilateral intraventricular injection with a total dose of 0.48 mg of cycloheximide,together with 60 µCi of ^{3}H-leucine,in a total volume of 50 µl,while under fluothane anesthesia.Rats B received,under the same conditions, 0.48 mg of cycloheximide, 2.4 mg of chloramphenicol,and 25 µCi of ^{14}C-leucine. Both A and B rats were killed 50 minutes later by cervical dislocation,and their brains pooled and processed together.This experiment was performed twice: 8 different subcellular fractions were examined in the first one,and 11 in the second one.

Although we have described recently one of the best methods available at present for the preparation of nerve ending fractions (6),the nature of the protein-synthesizing system under study has prompted this author to try to develop new technical improvements capable of freeing the synaptic membrane preparation from any mitochondrial vestige.This has been achieved by introducing flotation techniques in the two sucrose gradients involved

in the procedure.The rationale for this change is that mitochondria have higher density but lower sedimentation rate than synaptosomes and synaptosomal ghosts.Then,if the crude mitochondrial pellet,or the synaptosomal lysate, are placed in the gradient in a layer having a density greater than that of synaptosomes (in the 1.2 M sucrose layer of the first gradient),or synaptosomal ghosts (in a newly-introduced 1.1 M sucrose layer in the second gradient) respectively,but lower than that of mitochondria,when the centrifugal field is applied mitochondria will move downwards and synaptosomes and synaptosomal ghosts upwards,thereby avoiding any trapping and contamination.Synaptosomal membranes prepared in this way do not contain any detectable cytochrome oxidase activity,and can be considered free of mitochondrial elements.All the details of this new procedure will be given in a forthcoming paper (7). Crude nuclei were purified by pelleting them through 2.2 M sucrose.The endoplasmic reticulum fraction was taken as the material precipitating from the post-mitochondrial supernatant between 10,000g and 105,000g.The cell body mitochondria were obtained,free from remaining nuclei and capillaries,over a 1.4 M sucrose layer added to the first sucrose gradient.Myelin was collected in a 0.32-0.6 M interface in the first sucrose gradient.In this same sucrose gradient the material sedimenting at the 0.6-0.8 M interface was collected as a crude plasma membrane fraction,and further purified by means of a discontinuous cesium chloride gradient (6).Synaptic vesicles were obtained at a 0.32-0.6 M interface after applying a 105,000g synaptosol pellet to a new discontinuous sucrose gradient.Finally,a junctional complex-enriched fraction was prepared by extracting the synaptosomal plasma membrane fraction with 0.25% Triton X-100,as previously described (2,6).

Both ^{3}H and ^{14}C protein radioactivities,and the ^{3}H protein specific activity of the different fractions were measured as previously described (1), but,in view of the low level of radioactivity in protein and the high amount of non-incorporated amino acid to be washed off,four 10% trichloroacetic acid washes were used,and the lipid extraction was performed by either ethanol-

ether (1:1) or chloroform-methanol (2:1),followed in both cases by acetone and ethanol-ether (1:3) washes.No differences were found between the two lipid extraction methods described.

RESULTS AND DISCUSSION

The rationale for the experimental approach used stems from the fact that the chloramphenicol-sensitive protein synthesis in the synaptic junction represents only a very small portion of the total protein turnover in the synaptosomal membrane (2).Then I did not expect to see any measurable quantitative changes by only injecting chloramphenicol to the rats while keeping intact the bulk of neuronal protein synthesis and the axoplasmic transport mechanisms.Besides,the use of a double isotope method provides an efficient and reliable control for different efficiencies in injection and distribution of the radioactive precursors,and permits an objective estimate of the effect of chloramphenicol as calculated from the decrease in the ratio of ^{14}C to ^{3}H protein radioactivities relative to that of the microsomal fraction,which may be taken as an absolute standard for pure cycloheximide-sensitive protein synthesis.

Table 1 displays the results of the experiments performed.It is apparent from the data given that chloramphenicol does affect protein synthesis in the synaptosomal membrane,and more specifically in the synaptic junction (the 0.25% Triton X-100-insoluble residue (2,6)).The fact that only the two mitochondrial fractions are significantly affected by chloramphenicol,besides the synaptic membrane,strongly suggests that chloramphenicol is really acting as a protein synthesis inhibitor,and not by some other mechanism.Furthermore the finding that the $^{14}C/^{3}H$ ratio is sensibly the same in both the synaptosol and the synaptic vesicles as it is in the microsomal fraction implies that chloramphenicol does not interfere with axoplasmic transport,and that consequently differences found in the synaptic area should be attributed to failure in local synthesis.Finally I want to stress that although the impro-

TABLE 1. RELATIVE ADDITIONAL INHIBITORY EFFECT OF CHLORAMPHENICOL ON AMINO ACID INCORPORATION INTO RAT BRAIN SUBCELLULAR FRACTIONS IN VIVO.

Fraction	Disintegrations/ 100 min. ^{3}H	^{14}C	$^{14}C/^{3}H$ ratio	Relative additional inhibition by chloramphenicol %		^{3}H specific activity CPM/mg
Endoplasmic reticulum	1,469,822	341,807	0.233	= 0	(= 0)	584
Cytosol	303,730	61,897	0.204	12.4	(9.7)	106
Nuclei	44,758	8,785	0.196	15.9	(16.1)	-
Myelin	55,042	12,967	0.236	0		80
Plasma membrane	41,892	9,742	0.233	0		-
Cell body mitochondria	114,096	12,230	0.107	54.1	(54.3)	406
Synaptosol	50,872	12,092	0.238	0	(2.6)	58
Synaptic vesicles	13,296	2,822	0.212	9.0		86
Synaptic mitochondria	26,146	3,000	0.115	50.6	(61.7)	227
Synaptosomal membrane	258,220	19,887	0.077	67.0	(68.5)	286
Junctional complex	403,542	21,145	0.052	77.7	(79.4)	-

Rat brain subcellular fractions were prepared as described in the text. A portion of each fraction (0.5 to 10 mg) was processed for radioactivity measurement, and also for protein specific activity calculation whenever there was extra material available. The $^{14}C/^{3}H$ ratio was calculated, and an estimate of the additional inhibitory effect of chloramphenicol on protein synthesis in each fraction was calculated assuming no effect of chloramphenicol on the endoplasmic reticulum fraction. The specific activity of ^{3}H in proteins of some fractions, given in the table, has been corrected to a ^{3}H counting efficiency of 36%, as in our previous single-label experiments (2). It gives us an idea of the residual level of protein synthesis after the cycloheximide injection. Only the results of the second experiment are given here in full detail. The percent inhibition values for the first experiment are given in parentheses to the right of the corresponding value in the second experiment.

vements in the procedure make us feel safe concerning mitochondrial contamination,the fact that the effect of chloramphenicol is significantly more intense in the synaptic membrane than in both types of mitochondria definitively rules out the contamination hypothesis as an explanation of our previous and present results.

I would like to remark that only those subcellular fractions of a demonstrated purity have been listed in Table 1.Even so the 15% inhibition registered in the nuclear fraction could be accounted for by mitochondrial contamination (mitochondria do appear in electron microscopic studies even after purifying the nuclei through high density sucrose layers (8)); equally the small inhibition found in the synaptic vesicles could be adscribed to very small synaptic membrane fragments.

I must also note at this point that the intraventricular injection of chloramphenicol invariably induces in the rat a status of drowsiness, with ataxia and occasional seizures.One might be tempted to relate these symptoms to the protein synthesis inhibition found.But as Dunn (9) has shown in his studies on the effect of electroshock on brain protein synthesis,it is not the accompanying convulsions but the electroshock itself which is responsible for the protein synthesis inhibition.By analogy, we may assume that the post-chloramphenicol syndrome is a consequence of the metabolic failure of the mitochondria and synaptic junction,and not just its cause.On the other hand it is most logical to assume that impairment of mitochondrial and synaptic protein synthesis will severely disturb neuronal (and glial) function,giving rise to symptoms such as the ones described.

Our present knowledge about the effects of chloramphenicol on mammalian systems has been recently reviewed by Weisberger (3).Apparently chloramphenicol is capable of inhibiting protein synthesis in maturing or actively proliferating mammalian cells,and also in the case of newly induced antibody synthesis (10).In those cases,chloramphenicol would act by interference with a fast-turnover mRNA induced in response to the special metabolic conditions

of the cell (3).Although in the rats used one should assume that brain cells are already mature and differentiated,some functional aspects of the nervous system concerning information processing and adaptative responses to changing environmental conditions might ressemble the induction of new antibodies in immunocytes,including the appearance of special types of RNA at the synaptic level.We are currently investigating whether the synaptosomal membrane RNA (6) has some properties in common with the mRNA induced by antigenic stimulation,including structural features responsible for the chloramphenicol sensitivity.

ACKNOWLEDGEMENTS

This work was done while I was holding a Long-term Fellowship from the European Molecular Biology Organization.I am indebted to Ing.Birgitta Karlsson for superb technical assistance,and to Prof.Charles L.Perrin for critical review of the manuscript.This work was supported by grant no. B 72-13X-86-08B from the Swedish Medical Research Council to Prof.Holger Hydén, whose hospitality and continuous support are gratefully acknowledged.

REFERENCES

1. Ramirez,G.,Levitan,I.B.,and Mushynski,W.E., FEBS Letters 21,17 (1972).
2. Ramirez,G.,Levitan,I.B.,and Mushynski,W.E., J.Biol.Chem. 247,5382 (1972).
3. Weisberger,A.S.,in "The Interaction of Drugs and Subcellular Components in Animal Cells" (P.N.Campbell,ed.) ,p.133, J.& A.Churchill,London(1968).
4. Squire,L.R.,and Barondes,S.H., Proc.Nat.Acad.Sci.USA 69,1416 (1972).
5. Firkin,F.C.,and Linnane,A.W., Exptl.Cell Res. 55,68 (1969).
6. Levitan,I.B.,Mushynski,W.E.,and Ramirez,G., J.Biol.Chem. 247,5376 (1972).
7. Ramirez,G.,Perrin,C.L.,and Hansson,H.-A.,in preparation.
8. Badr,G.,personal communication.
9. Dunn,A.,Brain Research 35,254 (1971).
10. Ambrose,C.T.,and Coons,A.,J.Exptl.Med. 117,1075 (1963).