"IN VITRO" LABELLED NEUROTRANSMITTERS RELEASE FOR THE STUDY OF NEUROTOXINS.

Maria A.P.Camillo (1); Lanfranco R.P.Troncone (2) e José R.Rogero (1)

(1) Supervisão de Radiobiologia.IPEN-CNEN/SP ;
Travessa R, 400 - Cid. Universitária - 05508.900
São Paulo - SP - Brasil

(2) Laboratório de Farmacologia.INSTITUTO BUTANTAN
Av. Vital Brasil 1500 - 05503.900
São Paulo - SP -Brasil

ABSTRACT

There is an increasing concern in the replacement of "in vivo" by "in vitro" methods in Pharmacology. Looking for a method which involves the most of the physiological aspects related to neural functions, a superfusion system designed to evaluate "in vitro" neurotransmitter release from brain striatal tissue is here described. The method is based on the basal and stimulated release of pre-loaded tritium-labelled neurotransmitters. This procedure bears an active uptake/release function which is fairly changed by membrane polarisation state, ion channel activation and enzymatic activity, as well as other still unknown steps involved in neurotransmission. Calcium dependency of dopamine (DA) and acetylcholine (Ach) release induced by high potassium depolarisation or glutamate (Glu) stimulation was demonstrated employing calcium-free (+EGTA) superfusion or lanthanum/cadmium addition. Glutamate stimulation involved NMDA receptors since magnesium or MK801 blocks stimulated release. Uptake of DA and Ach was evidenced by using bupropione or hemicolinium-3. Presynaptic inhibition of Ach release was evidenced by physostigmine-induced inhibitions of acetylcholinesterase.

INTRODUCTION

During the last two decades neurotoxins have attracted growing attention. A large number of these toxins produced by microbes, spiders, scorpions , snakes or marine animals exhibit extreme specificity of action and, hence, they have proved to be valuable tools in neurobiology [1]. Much progress in neurochemistry and neurophysiology is due to the successful use of toxins as dissecting instruments for elucidating structural and physiological functions of neuronal systems.

In the last few years we have been developing studies to evaluate the interference of toxins isolated from venoms of Brazilian snakes and spiders in neurophysiological process. We have chosen a method which involves the most of the physiological aspects related to neural functions, i.e., a chopped brain tissue previously loaded with tritium-labelled neurotransmitters and superfused with incubating medium, in a system designed to evaluate the neurotransmitter release .The purpose of this account is to describe some aspects of its standardisation.

MATERIAL AND METHODS

Neurotransmitter release was assessed as described by Marien et al (1983) [1]. Briefly, adult male Wistar rats were killed by decapitation and the striatum was dissected and placed in ice-cold Krebs-Ringer-bicarbonate buffer (KRB) with the following mM composition: NaCl 118, $NaHCO_3$ 25, KCl 4.8, $CaCl_2$ 1.2 and Glucose 10, pH 7.3. As glycine has been proven to effect glutamate receptor effectiveness, this amino acid 10 uM was added to KRB. When high K^+ concentration was employed as stimulus, 1.2 mM $MgSO_4$ was also included in KRB. Constant gassing with CO_2/O_2 was maintained. Striatal tissue was then shopped twice in a McIlwain tissue shopper set to cut at 250 µm to form prisms. Shopped tissue was dispersed with a pipette and washed twice in 20 ml ice cold KRB; it was then transferred to a beaker containing 3 ml KRB plus 15 µl of [^{3}H]-Dopamine ([^{3}H]-DA) (New England Nuclear /specific activity 36.9 Ci/mMol) and 10 µM pargiline to prevent dopamine breakdown or 15 µl of [^{3}H]-Choline (New England Nuclear/ specific activity 88.7 Ci/mMol) and maintained at 37^0 C for 20 minutes to allow uptake. Tissue was then filtered and washed twice with normal ice cold KRB and distributed in ten superfusion chambers with internal volume of 0.25 ml. Superfusion was performed at a rate of 0.35 ml/minute with a ten channel peristaltic pump, during 60 minutes in order to achieve a stable baseline of [^{3}H]-neurotransmitter release. After this time, three successive baseline samples were collected with an adapted home-made collector, at three minute intervals. Superfusion with stimulating agents followed in the fourth three minute interval and lasted two minutes. A 20 mM K^+ KRB (NaCl reduced isomolarly) was used to depolarise striatal tissue and induce release. Monosodium glutamate 100 µM was employed as stimulus also for two minutes. A second identical stimulus was performed in the 11th sample and six more samples were afterwards collected. In the 19th interval , tissue was perfused with HCl 0.1 N for two periods of three minutes to induced release of total amount of [^{3}H]-neurotransmitter still present in the tissue. Test substances were introduced in superfusion medium in the 8th interval: Hemicolinium-3 100 µM ; Physostigmine 100µM; MK801 100 µM or Bupropione 10 µM.

When calcium-dependence of K+-stimulated release was tested, this ion was omitted in the buffer (KRB). Cadmium 100 µM and Lanthanum 100 µM were used in Mg+-free KRB buffer to test calcium-dependence of glutamate-stimulated release. The effect of Magnesium 2.1 mM in glutamate-stimulated neurotransmitter release was tested in ordinary KRB solution.

Results are expressed as fractional release, i.e., percent of [^{3}H]-neurotransmitter released over total contained in the tissue at the time of release. Effect of drugs were evaluated by the S2/S1 ratio, between stimulated release in the presence of drug (S2) and control stimulated release (S1). Basal release in the presence of drug (B2) and control basal release (B1) were used to evaluate the effects of drug on basal release using B2/B1 ratio. S(n) is calculated subtracting B(n) from the total release obtained under stimulation. B1 corresponds to the mean of the three samples that preceded stimulation and the last fractional release before S2 was considered as B2. Figure 1 depicts a typical result obtained in these procedures.

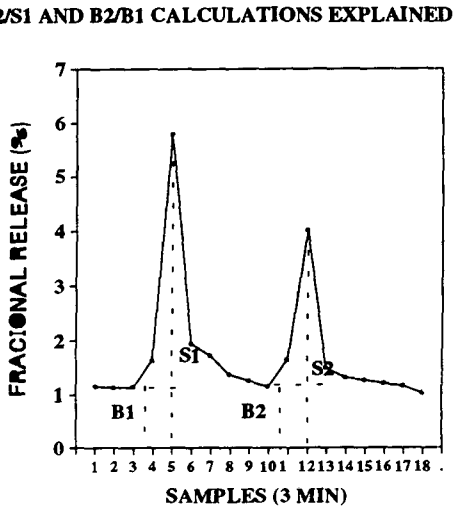

FIGURE 1- Typical superfusion result showing B1, B2, S1 and S2 calculations.

RESULTS

Results are explained in legends.

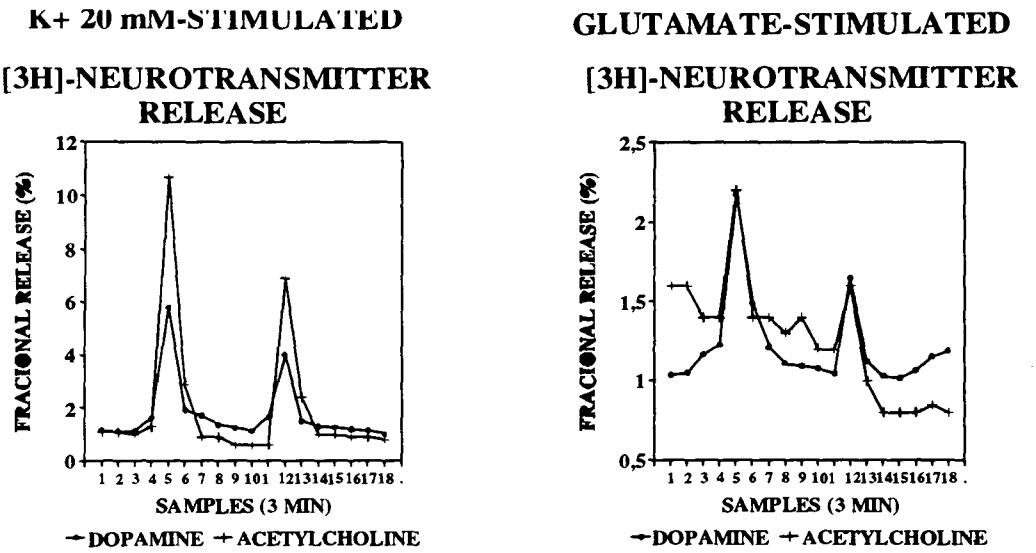

FIGURE 2 -Typical profile of Dopamine and Acetylcholine releases induced by 20 mM K^+ or 100 µM glutamate in a Mg^+-free superfusion fluid. Potassium induces higher Ach release than DA release. Glutamate induces similar releases on Ach and DA.

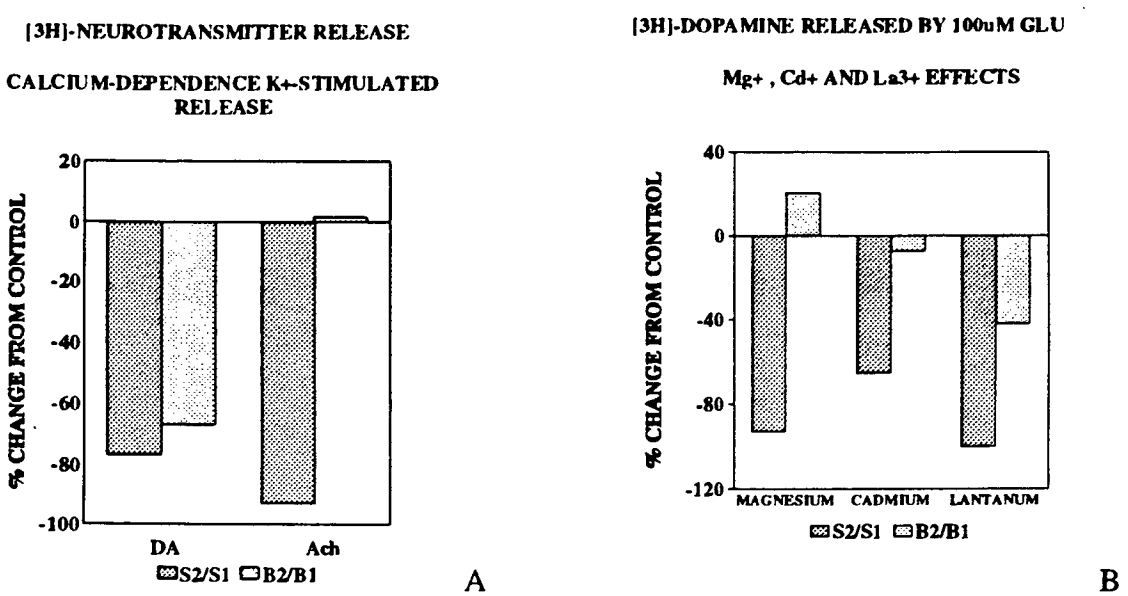

FIGURE 3- Calcium dependence in [^{3}H]-neurotransmitter release. A) K^+ -stimulated release. B) [^{3}H]-DA release stimulated by glutamate 100 µM -effect of calcium-competing ions and magnesium. Note that basal DA release is sensitive to external calcium but basal Ach is almost insensitive. Both neurotransmitters depend on calcium to be released by K^+ stimulation. Mg^+ blocks glutamate-stimulated release and calcium-competing ions impairs it to.

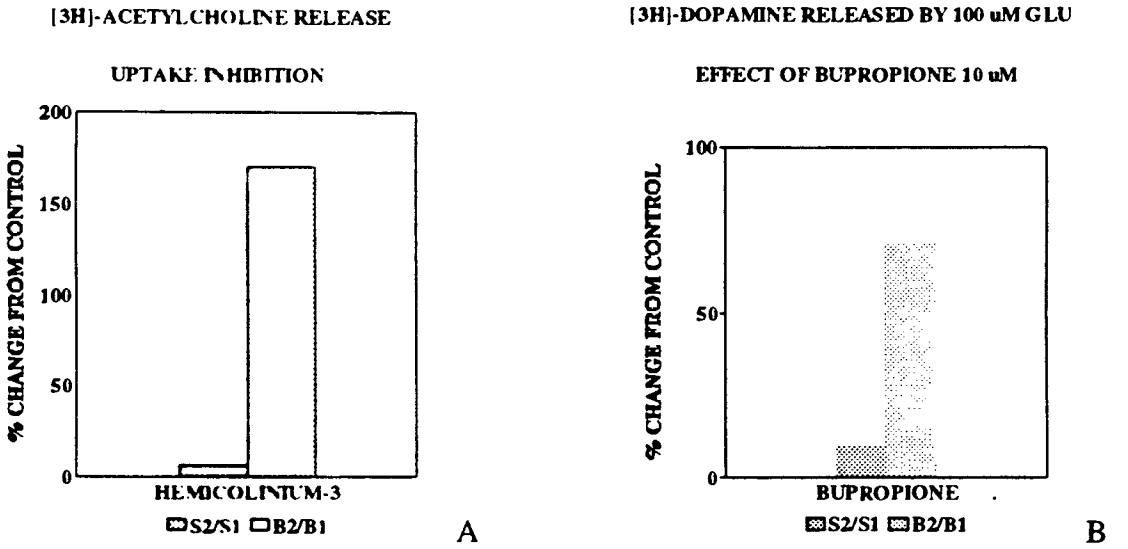

FIGURE 4- Inhibition of the [^{3}H]-neurotransmitters uptake. A) Effect of the addition of Hemicolinium-3 100 µM in the [^{3}H]-Ach release. B) Effect of bupropione 10 µM on glutamate stimulated (100 µM) [^{3}H]-DA release. Both uptake inhibitors enhance basal release without changing stimulated release.

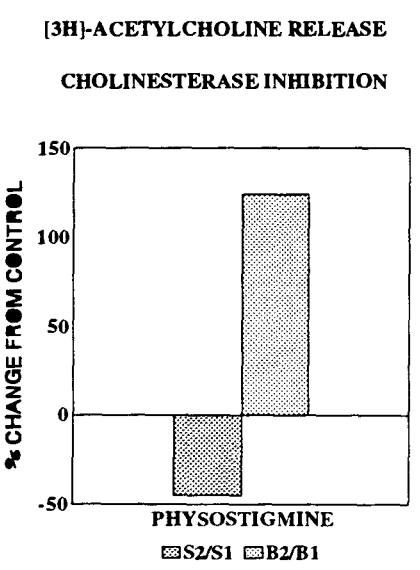

FIGURE 5- Effect of Physostigmine 20 μM stimulated release of [^{3}H]-Ach. Note the enhanced basal release and consequent autoreceptor-mediated inhibition of stimulated release.

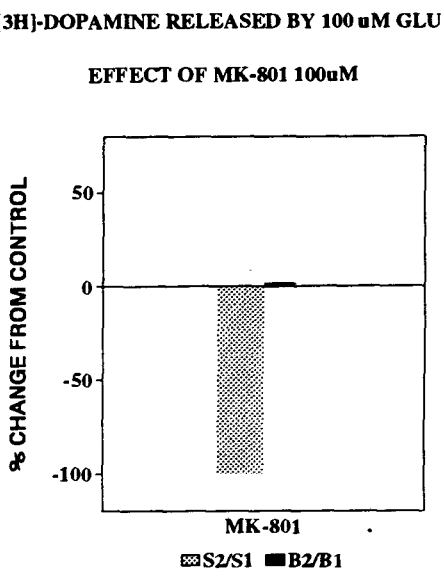

FIGURE 6- Effect of MK.801 in the glutamate stimulated release (100 μM) of [^{3}H]-DA. Note strong inhibitory action on glutamate stimulated release.

DISCUSSION

Neurotransmitter release is the resulting event of a complex and still unknown biochemical process. The amount of chemical messenger released depends on ionic currents generated by depolarisation, receptor activation of channels, presynaptic autoreceptors and heteroreceptors as well as and transmitter uptake and metabolization. In addition, a series of chemical steps involved in vesicular exocytosis is still waiting an appropriate explanation which should consider the growing amount of data involving vesicle-specific proteins, phosphorilation, calcium dependence and so on. The evaluation of neurotransmitter release is, therefore a valuable way to assess the effects of chemicals on neural functions. Also the relationship of inhibition or facilitation in neural circuits are suitable to be described by this method as well as the pharmacological characterisation of receptor subtypes involved.

The choice of the striatum among several other relatively large neural structures, was motivated by the good neuroanatomical and biochemical background already available. The presence of dopamine (DA) terminals and virtual absence of noradrenaline renders this structure specially suitable for [^{3}H]-DA release without having to demonstrate that the released label is not on noradrenaline which biosynthesis is derived from DA. Also, an intrinsic cholinergic neurone is known as well as GABA- and glutamate-releasing neurone terminals. Neuropeptides as substance P and CCK are also present.

Our studies revelled that labelled DA and choline are actively taken up by neural cells in striatal chopped tissue and incorporated in the releasable neurotransmitter pool. Stimulated release can be achieved by mild depolarisation with K^+ 20 mM which opens voltage-sensitive calcium channels triggering the release machinery. The absence of extracellular calcium blocks virtually all stimulated release of DA and acetylcholine (Ach), as represented in figure 3.

Inactivation of released neurotransmitter was also assessed. For DA the uptake is the main inactivating process. The addition of bupropione, a DA uptake blocker, induced a striking increase in basal release with almost no change in glutamate-stimulated release (figure 4). By its turn Ach is

inactivated by enzymatic breakdown and the resulting choline is selectively taken up. Hemicolinium-3, a choline uptake blocker, also induces a strong increase in basal 3H- release without changes in K^+ stimulated release (figure 4A). Physostigmine, an acetylcholinesterase inhibitor, blocked Ach breakdown and no 3H-choline was generated for uptake, therefore, an increase in basal release of 3H-Ach was observed (figure 5). Also, blockade of the inactivation process of Ach revelled a smaller stimulated release. This could be explained by a presynaptic autoreceptor stimulation impairing further Ach release.

Glutamate (Glu), the main excitatory amino acid neurotransmitter induces a dose-dependent DA and Ach release in striatum. Glu-receptors are prominent calcium-preferring ion channels. Three receptor types are currently accepted: NMDA, AMPA/kainate and quisqualate selective receptors [3]. NMDA is known by its Mg^+ and MK801 sensitivity. Kainic and quisqualic acid were much weaker DA release inducers. We were able to block Glu-stimulated release of DA with MK801 (figure 6) and Mg^+ ions, suggesting an NMDA-governed process. Ach can be released by Glu in the absence of Mg^+ but addition of this ion or MK801 were not tested yet.

Since the omission of Mg^+ is mandatory for Glu stimulation, the absence of calcium can not be practised since the stability of the neural cell seems to be lost and no work can be performed. To challenge the calcium-dependence of Glu-stimulated release, the addition of cadmium or lanthanum proved to be an useful strategy. Here we observed that these two ions blocked Glu-stimulated DA release (figure 3B).

CONCLUSION

The method described here represents a valuable choice to study neurotoxins and its actions in a physiological neurotransmitter releasing activity for DA and Ach The main theoretical issue can be summarised by considering it as an intermediary system standing between the molecular interactions studied in receptor binding procedures and complex multi-functional interactions seen in behavioural studies. It conjugates the simplicity of isolated neural circuits and the importance of a functional assay in opposition to purely chemical interactions which can easily induce to mistakes as those observed with drugs presenting high affinity for specific binding sites but low pharmacological effect in the living tissue. The most of the equipment was adapted from low cost trivial laboratory materials. Chemicals involved are also of low cost unless labelled transmitters. The disadvantage of radioactive handling is fairly compensated by the high sensitivity and specificity achieved.

Financial support FAPESP 89/0910-2 (LRPT).

REFERENCES

[1] Marien, M., Brien, J., Jhamandas, K.. Regional release of 3H-dopamine from rat brain "in vitro": effects of opioids on release induced by potassium, nicotine and L-glutamic acid. **Canadian Journal of Physiology and Pharmacology**, v.61, p. 43-60, 1983.

[2] Mebs, D. and Hucho, F.. Toxins acting on ion channels and synapses. In: **Handbook of Toxinology**. Shier,T. and Mebs,D. (eds). Marcel Dekker Inc. p.493-599, 1990

[3] Krebs, M.O.; Desce, J.M.; Kemel, M.L.; Gauchy, C.; Godeheu,G.; Cheramy, A.; Glowinski, J..Glutamatergic control of dopamine release in the rat striatum: Evidence fro presynaptic NMDA receptors on dopaminergic nerve terminals. **Journal of Neurochemistry**,v.56,p.81-85, 1991