Volume 263, number 1, 61–65 FEBS 08297 April 1990

# Tetanus toxin-induced protein kinase C activation and elevated serotonin levels in the perinatal rat brain

Jose Aguilera*,+, Lluis A. Lopez+ and Ephraim Yavin*

*Department of Neurobiology, Weizmann Institute of Science, Rehovot, Israel and +Department of Biochemistry and Molecular Biology, Universidad Autonoma de Barcelona, Cerdanyola, Spain

Received 11 December 1989; revised version received 6 February 1990

A single intracerebral injection of tetanus toxin (TeTox) is able to produce a time-dependent translocation of $Ca^{2+}$-phosphatidylserine-dependent protein kinase C (PKC) in close-to-term rat brain. TeTox-triggered translocation of PKC is dose- and time-dependent, can be prevented by tetanus antitoxin, and does not occur upon administration of toxin fragments B and C. TeTox-triggered PKC translocation is accompanied by a time-dependent increase in brain serotonin (5-HT). Increase of brain 5-HT is independent of monoamine oxidase inhibition by pargyline. Phorbol ester and TeTox cause a significant increase in serotonin while H-7, a kinase inhibitor, does not affect serotonin levels but abolishes the effect of TeTox. Gangliosides prevent TeTox-triggered 5-HT increase. The data are consistent with the possibility that TeTox acts effectively on the serotonergic innervation, presumably in conjunction with PKC to cause accumulation of serotonin.

Tetanus toxin; Serotonin; Protein kinase C; Ganglioside; Brain development

## 1. INTRODUCTION

Tetanus toxin is probably the oldest neurotoxin recorded in humans and still a most serious health problem in some underdeveloped countries [1]. In spite of extensive research carried out for nearly 3 decades [2], little information is available about the molecular mechanism underlying its toxicity, or the nature of the target(s) implicated in its retrograde axonal transport, synaptic traverse and internalization in vivo [3–5]. TeTox is composed of a heavy chain (100 kDa) and a light chain (50 kDa) held together by a disulfide bond [6,7]. Moderate proteolytic cleavage by papain, results in two nontoxic fragments [8] of molecular mass 45 kDa (fragment C) and 100 kDa (fragment B).

The strong affinity of TeTox towards the nervous tissue has been attributed to a binding component which consists of a disialo- or trisialoganglioside [9]. Nevertheless gangliosides represent probably only one constituent of a more complex receptor which, upon interaction with the toxin causes internalization of the latter [5,10–12] and/or a blockade of a $Ca^{2+}$-dependent $K^+$-stimulated neurotransmitter release process [13].

In our search for a possible molecular target to account for the in vivo action of the toxin, we have recently reported a TeTox-dependent translocation and down-regulation of protein kinase C [14]. Likewise we have shown that TeTox causes an increase of serotonin synthesis in the central nervous system [15]. In this report we present evidence suggesting that PKC translocation and serotonin accumulation predate the clinical symptoms and neurotoxicity, and may be two closely associated phenomena.

## 2. MATERIALS AND METHODS

### 2.1. *Tetanus toxin*

Highly purified tetanus toxin preparations were kindly provided by Dr W. Habig (Laboratory of Bacterial Toxins, FDA, Bethesda, MD) and Dr C. Montecucco (University of Padova). The solutions contained approximately $10^7$ mouse lethal doses (MLD)/mg toxin and showed one band on SDS gel electrophoresis under nonreducing conditions, as previously described [16].

### 2.2. *Animal manipulation*

Gravid albino rats (Wistar strain) at 20 days gestation were lightly anesthetized by intramuscular injection of 0.1 ml/200 g of body weight of a mixture (1:1 v/v) of ketamine (25 mg/kg; Parke-Davis, UK) and Rumpon 2% (Bayer, FRG). An abdominal midline incision was performed and the uterine horns exposed.

Tetanus toxin, in 2 μl $P_i$/saline buffer was injected with a Hamilton syringe (10 μl) by passing through the uterine wall toward the fetal brain skull to a point 1 mm below the fontanella and 3 mm deep, into the ventricle. A similar injection protocol without anesthesia was carried out using neonatal rats. As indicated, TPA, H-7 (Sigma, St. Louis, MO), gangliosides (Fidia Res. Lab., Abano

*Correspondence address:* E. Yavin, Department of Neurobiology, Weizmann Institute of Science, 76100 Rehovot, Israel

*Abbreviations:* H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; GM1, galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide; GD1b, galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl-*N*-acetylneuraminyl)galactosylglucosylceramide; BBG, bovine brain ganglioside mixture; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; PKC, $Ca^{2+}$-phosphatidylserine-dependent protein kinase C; TeTox, tetanus toxin

*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/90/$3.50 

Terme, Italy), tetanus antitoxin (Sclavo, Wayne, NJ) or TeTox fragments B and C (Calbiochem) were injected by the same route in 2 μl final volume.

After treatment, fetuses were returned to the abdominal cavity and at desired times were delivered, resuscitated, and killed by decapitation. Brains were rapidly removed and immediately subjected to further analysis. Administration of pargyline (Sigma, St. Louis, MO) was done by intraperitoneal injection.

2.3. *Protein kinase C analysis*

Brain tissue was homogenized with a Polytron apparatus using 9 vols of buffer consisting of 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 2 mM dithiothreitol, 2 mM phenylmethylsulphonylfluoride, and 10 μl/ml leupeptin, aprotinin, and pepstatin A. The homogenate was centrifuged at 100000 × *g* for 1 h, to obtain both soluble and particulate fractions. The latter was resuspended to the original volume using the buffer as above, to which 0.3% Triton X-100 (final concentration) was added. After 1 h incubation at 4°C, the Triton X-100-treated particulate fraction was centrifuged at 100000 × *g* for 1 h, to obtain the Triton X-100 soluble fraction [17]. Each of the ensuing supernatants was mixed with a slurry of DEAE-cellulose (0.75 ml) and after shaking for 15 min, samples were centrifuged for 1 min at 20000 × *g* in an Eppendorf microfuge. The supernatant was discarded and pellet washed 4 times with 0.75 ml of buffer containing 20 mM NaCl in 20 mM Tris-HCl, pH 7.5, and 2 mM EDTA, 0.5 mM EGTA, 2 mM dithiothreitol and 0.1% Triton X-100. Elution of PKC was accomplished at 4°C by two consecutive elutions, 0.5 ml each, of the above buffer supplemented with 200 mM NaCl. PKC was assayed in the presence of 0.04% Triton X-100 by measuring the incorporation of $^{32}P$ from [$\gamma$-$^{32}P$]ATP into lysine-rich histone (type III-S; Sigma, St. Louis, MO), as previously described [17]. Total PKC activity represents the combined activity of the soluble and detergent-extracted particulate fraction each determined independently and normalized per wet weight. Protein was determined according to Bradford [18].

2.4. *Serotonin determination*

Toxin-treated or untreated rat neonates were sacrificed by decapitation and whole cerebral hemispheres were excised and rapidly homogenized in 9 vols of 74% ethanol in water. The serotonin was extracted according to a published procedure [15] using a column containing a 0.4 × 2.5 cm Amberlite CG-50 ($H^+$, type II, 200–400 mesh, Prolabo) resin bed. The biogenic amines were retained at neutral pH and subsequently eluted with 5 ml of 0.2 N acetic acid. Serotonin was quantified by a fluorescent procedure described elsewhere [19]. Essentially similar results were obtained upon examination of close-to-term fetuses. All reagents used were of analytical grade.

## 3. RESULTS

A single intraventricular injection of 40 MLD of toxin produces a marked intracellular redistribution of PKC activity in the neonatal brain. As shown in Fig. 1, a substantial portion of PKC is translocated after 1 h from the cytosol to the membrane compartment. The reduction in activity in the soluble fraction, which amounted to about 25–35%, is entirely recovered in the membrane fraction suggesting that no apparent down-regulation of the enzyme occurred. Intraperitoneal injection of the TPA phorbol ester, produces an identical pattern of translocation as is also depicted in Fig. 1. The TeTox-induced translocation is time-dependent, as is illustrated in Fig. 2A. After 15 min and 30 min exposure, 38% and 44% of the enzyme activity, respectively, is associated with the membrane fraction

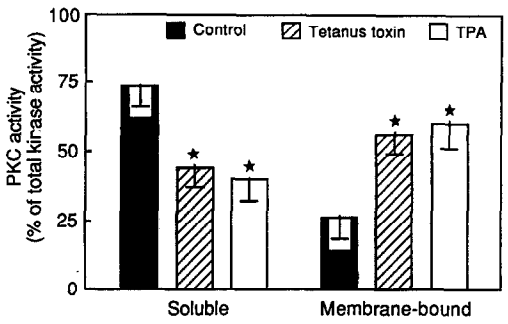


Fig. 1. Tetanus toxin and phorbol ester (TPA)-induced translocation of $Ca^{2+}$-phosphatidylserine-dependent protein kinase C. Rat neonates injected either intracerebrally with tetanus toxin (40 MLD/rat) in 2 μl medium (hatched bars) or intraperitoneally with 25 μl of 25 μM TPA solution (15 pmol/g body weight, 1.5 × $10^{-7}$ M) (open bars), were killed after 1 h or 10 min, respectively. Two separate groups of control animals were injected similarly with the corresponding vehicle, for the same time. Value given after the intracerebral injections of the vehicle (closed bars) was statistically comparable, to the intraperitoneal-sham-treated group. Values are expressed as per cent of total PKC activity. The total enzyme activity in the neonatal brain ranged between 200 and 250 U/mg protein. Each bar represents the mean ± SD of 3–5 individual animals. Student's *t*-test was used for calculating probability, which was significant (* $P < 0.005$).

compared to 31% in sham animals. By 60 min the enzymatic activity in the membrane fraction is doubled. The unique feature of TeTox in inducing translocation can be further appreciated from Fig. 2B, which clearly shows that even a limited dose (0.2 MLD) of TeTox can produce a significant effect on PKC distribution. The specificity of the toxin has been studied to some detail. First, as shown in Fig. 3, it is evident that the entire TeTox molecule is required in order to produce PKC activation. Second, neither fragment B which contains the toxic (albeit inactive) site [20], nor fragment C which contains the binding site, are able to produce translocation even at concentrations which are 100-fold greater than those used with TeTox. Premixing of the toxin with anti-TeTox, before application also entirely prevents the effect.

The question of neurotransmitter presynaptic specificity and the exact nature of the general impairment of neuronal circuits caused by intracerebral administration of tetanus neurotoxin is still an unresolved problem. Certain central symptoms of tetanus such as insomnia, parkinsonism, hyperthermia and hypertension seem to involve different monoaminergic systems [3]. In addressing this issue we were recently able to demonstrate, using adult rats, that of the various brain biogenic amines tested, serotonin exhibited the highest rise after intraventricular TeTox application [15,19]. Since serotonergic innervation is one of the first to appear during brain ontogeny [21] it was appropriate to investigate the levels of this biogenic amine following toxin administration during the perinatal phase of development.

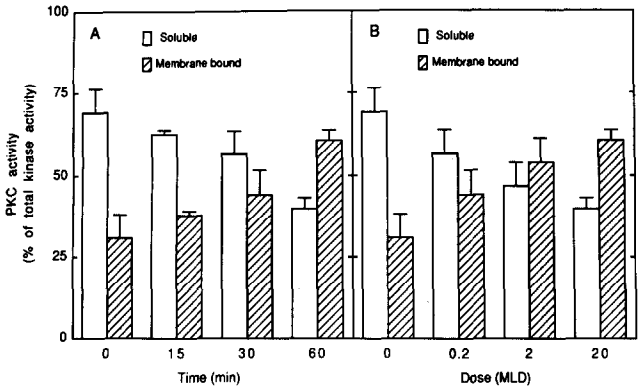


Fig. 2. Time- (panel A) and dose- (panel B) dependent protein kinase C translocation with a single intracerebral application of tetanus toxin. Intracerebral injection of 20 MLD (A) or varying doses (B) of tetanus toxin was performed in 2 $\mu$l volume as described in section 2. Animals (3/group) were killed at times designated (A) or after 1 h (B) exposure to the toxin and PKC activity (100–150 mU/mg protein) determined. The values expressed as percent activity are mean ± SD. The results analyzed using a Student's $t$-test were found significantly different between control and 20 min (20 MLD), and control and 2 and 20 MLD (panel B) for both soluble and membrane-bound PKC activities. The variance analysis of the means (one-way ANOVA) are also significant in all 4 groups ($P < 0.005$).

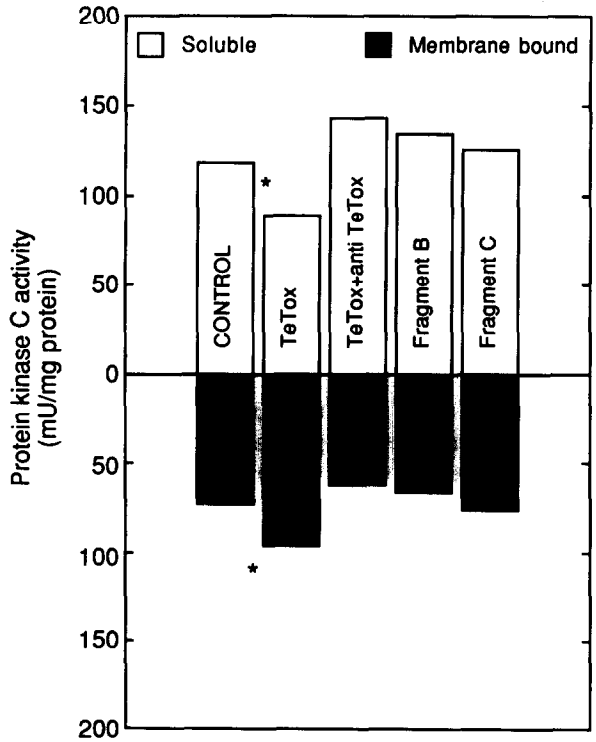


Fig. 3. Effect of toxin fragments and tetanus antitoxin on protein kinase C translocation. Rat neonates (3–4 per group) were injected and maintained for 1 h with nontoxic preparations of fragment B (0.5 $\mu$g protein/rat), fragment C (0.5 $\mu$g protein/animal) or with 40 MLD/animal in the absence or presence of excess amounts of equine antitoxin (administered by the same route). Values expressed as mU/mg protein are mean with a standard deviation of less than 10%. Student's $t$-test used to calculate probability in tetanus toxin treated animals was statistically significant with respect to the control or antitoxin groups (* $P < 0.05$).

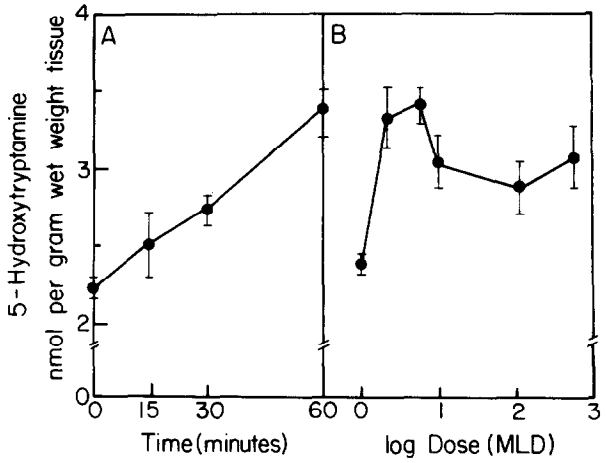


Fig. 4. Time- (panel A) and dose- (panel B) dependent 5-hydroxytryptamine accumulation after a single intracerebral application of tetanus toxin. Experimental details are essentially as detailed in Fig. 2, except that 7.5 MLD were used for the time course. The values expressed as nmol/g tissue ± SE were obtained from 3–4 animals.

As seen in Fig. 4, the increase in 5-hydroxytryptamine (5-HT) after intracerebral injection of TeTox (7.5 MLD) is time-dependent and linearly increases from a value of 2.28 ± 0.12 to a value of 3.37 ± 0.17 nmol/g brain after 1 h. This value can be obtained by injection of 2 MLD for 1 h but did not increase when 100 MLD or higher doses were injected.

In order to better understand the mechanism underlying the toxin-mediated serotonin increase, pargyline, the inhibitor of monoamine oxidase (MAO), was utilized [22]. Fig. 5 illustrates the changes in the levels of serotonin after administration of 5 MLD of TeTox. The data clearly show that serotonin is increased after 1 h in the presence of the toxin. Fig. 5 also shows that intraperitoneal administration of pargyline

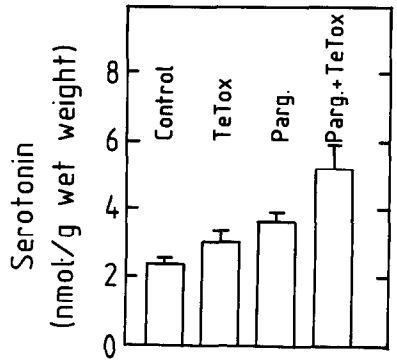


Fig. 5. Serotonin levels after tetanus toxin administration in the presence of pargyline. A single dose of TeTox (5 MLD) was given to 2-day-old rats by the intracerebral route in the absence or presence of pargyline (parg) given intraperitoneally (75 mg/kg) in 25 $\mu$l of $P_i$/saline buffer. After 1 h the animals (4–6 per group) were decapitated and serotonin determined as described in section 2. The results expressed as nmol/g brain ± SE were statistically significant using the Student's $t$-test.

Table I

Levels of 5-hydroxytrypamine in toxin-treated animals after exposure to various agents

| Stock solution | 5-HT nmol/g brain wet weight | |
|---|---|---|
| | Saline | Tetanus (5 MLD/animal) |
| Saline | 2.09 ± 0.04 | 2.92 ± 0.23* |
| Fragment B (0.25 mg/ml) | 2.10 ± 0.05 | N.D. |
| Fragment C (0.25 mg/ml) | 2.21 ± 0.17 | N.D. |
| GM1 (1 mg/ml) | 2.19 ± 0.09 | 2.53 ± 0.09* |
| GD1b (1 mg/ml) | 2.09 ± 0.17 | 1.68 ± 0.10** |
| BBG (1 mg/ml) | 1.92 ± 0.14 | 1.85 ± 0.14** |
| TPA (2 mg/ml) | 2.53 ± 0.07* | N.D. |
| H-7 (0.1 mg/ml) | 2.17 ± 0.15 | 2.05 ± 0.21** |
| Anti-TeTox (1 U/ml) | N.D. | 2.08 ± 0.00** |

The experiment was carried out essentially as detailed in Fig. 4, except that all compounds prepared at the noted concentrations were delivered by the intracerebral route in 2 μl final volume. Each value represents the mean ± SEM of 4–5 animals. Statistical evaluation ($P < 0.05$) between groups was determined by the Student's $t$-test with respect to naive (saline/saline) group (*) or toxin-treated (**) group. N.D. not determined

increases the levels of serotonin above the control values as expected from a compound which prevents degradation of biogenic amines in general [23]. Interestingly, serotonin levels continue to rise significantly from 2.28 ± 0.07 to 5.29 ± 0.7 nmol/g brain, when both TeTox and pargyline are administered concomitantly intraventricularly and intraperitoneally, respectively, into the animal.

The effect of TeTox on the levels of serotonin is highly specific as emphasized by a number of agents, the effects of which are summarized in Table I. In line with the lack of PKC translocation/activation, different fragments of tetanus toxin do not produce any considerable effect on serotonin levels. Furthermore, TeTox-induced serotonin increase is not seen in the presence of antitoxin and is prevented by the coadministration of GD1b or a mixture of bovine brain gangliosides and TeTox. The effect of GM1 may relate to its ability to bind TeTox in certain conditions [24]. Finally TPA, the specific activator of PKC, produces an increase in serotonin level while intracerebral administration of the H-7 kinase inhibitor abolishes TeTox-triggered serotonin accumulation as expected from a metabolic sequence which may involve protein kinase C [25].

## 4. DISCUSSION

In a recent study we have provided substantial evidence indicating that a single intraventricular injection of TeTox causes in the adult rat brain a marked activation followed by a down-regulation of PKC [14]. In a similar manner, we now demonstrate that a single dose of toxin as low as 0.2 MLD injected intracerebrally into the close-to-term or the neonatal rat causes within 15–30 min a substantial translocation of brain PKC. Under these experimental conditions, there are no behavioral changes in the animals and no signs of neurotoxicity, making remote the possibility of seizures as a reason for PKC translocation. In this context it is notable to recall that according to the definition, 1 MLD is the minimal amount of toxin necessary to kill a 20 g mouse in 4 days [16]. Thus, translocation of PKC takes place at subnanogram levels of toxin. This is to our knowledge the lowest level of toxin recorded which elicits a measurable biochemical response in tetanus. Furthermore, TeTox-triggered translocation is highly specific since papain digests of the toxin (e.g. fragments B and C) do not cause this effect and an antitoxin serum can prevent translocation.

The present data indicate that TeTox-triggered translocation of PKC is very similar to that obtained after intraperitoneal administration of the tumor promotor phorbol ester TPA. The effect of TPA occurs in vivo within 10 min presumably due to the rapid distribution of the lipophylic drug through the fetal organs. However, unlike the phorbol ester which acts at nanomolar concentration and is not selectively targeted to neuronal membranes, the TeTox-triggered PKC activation is highly specific to brain tissue.

The main thrust of this work has been to identify and characterize a possible neurotransmitter-target system(s) which may be affected at the time TeTox-triggered translocation/activation of PKC takes place. Biogenic amines, particularly serotonin, appeared to us as a potential candidate in the light of our previous studies, indicating an elevation of serotonin in the adult rat brain following intraventricular application of TeTox [15,19]. The developing brain was found suitable for these studies since it contains already fairly high levels of biogenic amines [21,26,30] including serotonin (i.e. 66% of the maximal adult values (see [19])). Furthermore the perinatal model was advantageous for the following reasons: first, administration of the toxin to the neonates did not require anesthetic procedures which may interfere with the translocation particularly during short-term studies. Second, the generalized symptoms of tetanus toxicity seen in the adult are practically nonexistent in the rat neonate perhaps because of an overall simplified neuronal circuitry, which does not induce secondary focal seizures. Evidently the early signs of spontaneous electroencephalographic activity in the rat appears first at 10 days postnatally [27].

After administration of the toxin, a time-dependent and dose-dependent change in the levels of serotonin is clearly evident. Accumulation of serotonin is in line with the general dogma that TeTox acts at presynaptic terminals to inhibit release of certain neurotransmitters, including excitatory ones [3,7]. Nevertheless, the additive effect of pargyline and TeTox on serotonin

levels (Fig. 5) indicates that the toxin affects the serotonergic system, perhaps through a second mechanism, i.e. the activation of tryptophan-5-monooxygenase. Phosphorylation of this enzyme by a calmodulin-dependent reaction has been indicated [28,29]; a protein kinase C-dependent activation has not been excluded. It is conceivable that TeTox may trigger tryptophan-5-monooxygenase phosphorylation by activating such a kinase, as much as it can act by inhibiting serotonin release.

The present findings indicate that PKC activation and serotonin accumulation may be closely related, based on the following arguments: first, TeTox causes PKC activation at extremely low doses, which is accompanied by a time- and dose-dependent 5-HT accumulation. Second, addition of antitoxin prevents TeTox-triggered PKC translocation, as well as accumulation of serotonin. The latter is also reduced in the presence of GD1b, the putative TeTox receptor. Third, TPA causes an increase in serotonin levels which strongly indicates a PKC-mediated response. A similar relationship has been established in brain slices subjected to phorbol esters [30,31]. Finally, H-7 prevents as expected from a protein kinase inhibitor the TeTox-triggered serotonin accumulation.

*Acknowledgements:* This research was supported in part by a grant from the Ministerio de Educacion y Ciencia (DGICYT, PB86-0679). J.A. is grateful to the Ministerio de Educacion y Ciencia (DGICYT) for enabling him a tenure stay at the Weizmann Institute of Science.

## REFERENCES

[1] Stanfield, J.P. and Galazka, A. (1984) Bull. World Health Org. 62, 647–669.
[2] Van Heyningen, W.E. (1961) J. Gen. Microbiol. 24, 107–119.
[3] Wellhoner, H.H. (1982) Rev. Physiol. Biochem. Pharmacol. 93, 1–68.
[4] Simpson, L.L. (1986) Rev. Pharmacol. Toxicol. 26, 427–453.
[5] Montecucco, C. (1988) in: Proc. Eighth Int. Conf. on Tetanus (Bizzini, B. and Nistico, A. eds) Pitagora Press, Rome, in press.
[6] Matsuda, M. and Yoneda, M. (1975) Infect. Immun. 12, 1147–1153.
[7] Bizzini, B. (1979) Microbiol. Rev. 43, 224–240.
[8] Helting, T.B. and Zwisler, O. (1977) J. Biol. Chem. 252, 187–193.
[9] Van Heyningen, W.E. (1974) Nature 249, 415–417.
[10] Yavin, E., Yavin, Z. and Kohn, L.D. (1983) J. Neurochem. 40, 1212–1219.
[11] Schmitt, A., Dreyer, F. and John, C. (1981) Naunyn-Schmiedeberg's Arch. Pharmacol. 317, 326–330.
[12] Lazarovici, P. and Yavin, E. (1986) Biochemistry 25, 7047–7054.
[13] Mellanby, J. (1984) Neuroscience 11, 29–34.
[14] Aguilera, J. and Yavin, E. (1990) J. Neurochem. 54, 339–342.
[15] Aguilera, J., Heredero, J. and Gonzalez-Sastre, F. (1987) Experientia 43, 410–412.
[16] Lazarovici, P., Tayot, J.L. and Yavin, E. (1984) Toxicon 22, 401–413.
[17] Louis, J.-C., Magal, E. and Yavin, E. (1988) J. Biol. Chem. 263, 19282–19285.
[18] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[19] Aguilera, J. and Gonzalez-Sastre, F. (1988) Biog. Amines 5, 405–408.
[20] Helting, T.B., Ronneberger, H.J., Vollerthun, R. and Neubauer, V. (1978) J. Biol. Chem. 253, 125–129.
[21] Lauder, J.M. and Bloom, F.E. (1974) J. Comp. Neurol. 155, 469–481.
[22] Schurr, A. (1982) Life Sci. 30, 1059–1063.
[23] Koe, B.K. and Weissman, A. (1966) J. Pharmacol. Exp. Ther. 154, 499–516.
[24] Helting, T.B., Zwisler, O. and Wiegandt, H. (1977) J. Biol. Chem. 252, 194–198.
[25] Nishizuka, Y. (1984) Nature 308, 693–698.
[26] Aitken, A.R. and Tork, I. (1988) J. Comp. Neurol. 274, 32–47.
[27] Bures, J. (1957) Electroencephalog. Clin. Neurophysiol. 9, 121–130.
[28] Yamauchi, T. and Fujisawa, H. (1980) FEBS Lett. 116, 141–144.
[29] Kuhn, D.M., O'Callaghan, J.P., Juskevich, J. and Lovenberg, W. (1980) Proc. Natl. Acad. Sci. USA 77, 4688–4691.
[30] Feuerstein, T.J., Allgaier, C. and Herting, G. (1989) Eur. J. Pharmacol. 139, 267–272.
[31] Wang, H.-Y. and Friedman, E. (1989) Eur. J. Pharmacol. 141, 15–21.