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European Journal of Pharmacology 296 (1996) 341–345

ejp

Short communication

Cyclosporin A affects functions concerning acetylcholine release of cholinergic *Torpedo* synaptosomes

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Received 6 September 1995; revised 21 November 1995; accepted 24 November 1995

Abstract

The effect of cyclosporin A was investigated on *Torpedo* synaptosomes. Cyclosporin A inhibits KCl-evoked acetylcholine release (up to 50% at 1 μM) and was inactive on acetylcholine release induced by a Ca^{2+} ionophore, A23187. Interestingly, when the synaptosomes were pretreated with cyclosporin A, this immunosuppressor did abolish the modulation of A23187-induced acetylcholine release produced by two other drugs, cetiedil (α-cyclohexyl-3-thienyl acetic acid 2-(hexahydro-1*H*-azepin-1-yl) ethyl ester, citrate salt) and MR16728 (*N*-(*N'*-hexamethylene imino)-propyl-phenyl-cyclohexyl-methyl acetamide, chlorhydrate), which were previously shown to be inhibitory and stimulatory, respectively. Moreover, cyclosporin A and MR16728 are competitive inhibitors of [^{3}H]cetiedil binding to purified synaptosomal presynaptic membranes (dissociation constant of 181.9 nM). These results suggest that presynaptic proteins involved in acetylcholine release (directly or indirectly through cyclophilin) are potential targets of cyclosporin A in *Torpedo* synaptosomes.

Keywords: Acetylcholine release; Cholinergic synaptosome; Cetiedil; Cyclosporin A

1. Introduction

Cyclosporin A, a cyclic peptide of fungal origin, is a potent immunosuppressive agent widely used to prevent allograft rejection. However, it has severe side effects. In particular, neurologic symptoms such as stupor, seizures and coma have been associated with high concentrations of cyclosporin A in the blood (Atkinson et al., 1984). Until recently, most of the work concerning the mechanisms of action of cyclosporin A focused on its effects on T lymphocyte functions. However, cyclosporin A inhibits a variety of other cellular functions such as the mechanism of secretion in different cellular types (for review, Hohman and Hultsch, 1990).

Until now, the effect of cyclosporin A on the secretion of acetylcholine from a neuromuscular junction was not documented. In the present study, we examined the action of cyclosporin A on acetylcholine release from nerve terminals (synaptosomes) isolated from *Torpedo marmorata* electric organ, a cholinergic tissue which is particularly rich in nerve terminals. Evoked acetylcholine release, which is elicited by a Ca^{2+} influx, was continuously followed in vitro by using a chemiluminescent procedure with choline oxydase (Israël and Lesbats, 1981). The use of two different stimuli, KCl depolarization or a Ca^{2+} ionophore, A23187, makes it possible to distinguish between agents whose action takes place before or after Ca^{2+} entry into the nerve terminal through voltage-dependent channels. Our previous effort was to characterize cholinergic agents which directly affect the evoked or spontaneous release of acetylcholine in *Torpedo* synaptosomes (Morot Gaudry-Talarmain et al., 1987; Moulian et al., 1993, 1994). We showed that cetiedil, a vasodilatator substance efficient in the treatment of sickle cells and with reported anticholinergic properties, inhibited acetylcholine release from *Torpedo* electric organ and synaptosomes regardless of the stimulus used to trigger it (an electric stimulation, a depolarization of the membrane with KCl, the addition of a Ca^{2+} ionophore). In contrast, its amide analogue MR16728 enhances A23187-induced acetylcholine release. In this study, we examined the effect of cyclosporin A on

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Elsevier Science B.V.
SSDI 0014-2999(95)00814-4

evoked acetylcholine release and on the inhibition or stimulation by cetiedil and MR16728, respectively. Moreover, after characterizing the binding of [^{3}H]-cetiedil on presynaptic membranes purified from *Torpedo* synaptosomes, we investigated the effect of cyclosporin A and MR16728 on this binding.

2. Materials and methods

Cetiedil (α-cyclohexyl-3-thienyl acetic acid 2-(hexahydro-1*H*-azepin-1-yl) ethyl ester, citrate salt) was synthesized according to Robba and Le Guen (1967). The cetiedil analogue MR16728 (*N*-(*N'*-hexamethylene imino)-propyl-phenyl-cyclohexyl-methyl acetamide, chlorhydrate) was synthesized in the laboratory of Prof. M. Robba (Caen, France). [^{3}H]Cetiedil was synthesized in the laboratory of J.-L. Morgat (C.E.N., Saclay, France). Stock solutions of cyclosporin A (Sandimmun, Sandoz, Switzerland), cetiedil and analogues (usually 5 mM) were diluted in distilled water; aliquots were stored at −80°C prior to use at the appropriate dilution. Other reagents were obtained from commercial sources.

Torpedo marmorata electric organ synaptosomes deriving from 25 g of tissue were isolated according to the method described by Morel et al. (1977). The effect of the chemical compounds on acetylcholine release from *Torpedo* synaptosomes was continuously followed using the choline oxydase chemiluminescent method of Israël and Lesbats (1981).

Presynaptic plasma membranes were purified from *Torpedo* synaptosomes according to the method of Morel et al. (1982). Briefly, synaptosomes were osmotically shocked and frozen and the total synaptosomal membranes were separated in different fractions on a discontinuous sucrose gradient. [^{3}H]Cetiedil binding was performed in a binding solution containing 5 mM Tris buffer, pH 7.2, and 150 mM NaCl. Purified presynaptic membranes, corresponding to 5 μg of protein, were incubated with different concentrations of [^{3}H]cetiedil in a volume of 100 μl; the specific radioactivity of [^{3}H]cetiedil was 7 Ci/mmol. The amount of [^{3}H]cetiedil bound to the membranes was determined after an incubation for 60 min on ice by a filtration technique. The samples were filtered on Whatman GF/C filters and washed with 15 ml of ice-cold binding solution. Filters were soaked before in the binding solution containing polyethylenimine (0.3%) for 2 h. Non-specific [^{3}H]cetiedil binding was determined using 225 μM non-radioactive cetiedil. Specific binding was taken as the difference between total binding and non-specific binding. For competition experiments, cyclosporin A or MR16728 was added to the binding

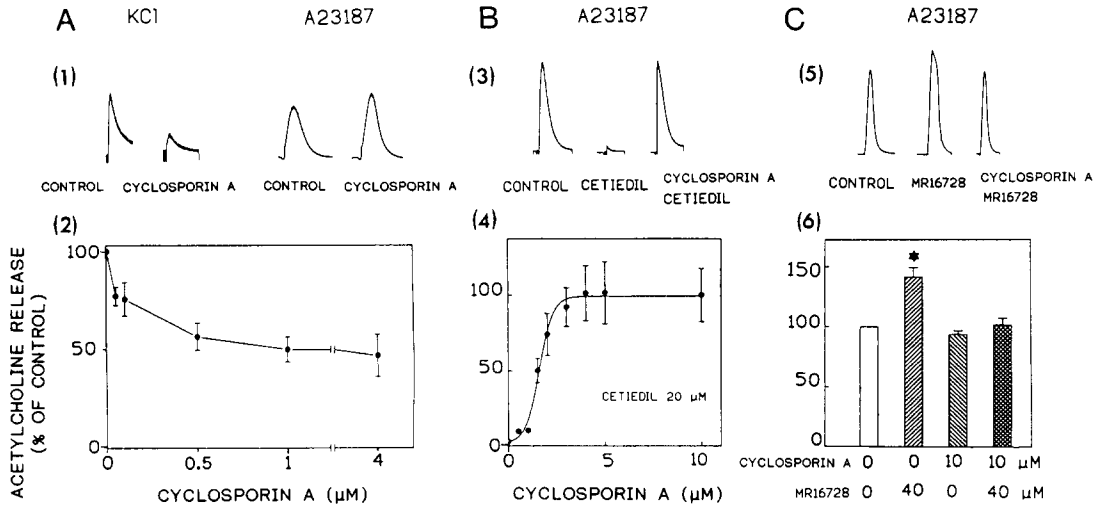

Fig. 1. A: Effect of cyclosporin A on evoked acetylcholine release. (1) Traces of a representative experiment are shown. Synaptosomes were incubated with cyclosporin A for 5 min. Then 4 mM Ca^{2+} was added. Acetylcholine release was induced by either 60 mM KCl (left) or 4 μM A23187 (right). Chemiluminescent recordings of evoked acetylcholine release were calibrated by injecting an internal acetylcholine standard (not shown). (2) The lower graph illustrates the inhibitory effect of cyclosporin A on KCl-induced acetylcholine release as a function of cyclosporin A concentration. Acetylcholine is expressed as a percentage of control release. Data are the mean ± S.E.M. from five independent experiments. B: Cyclosporin A prevents cetiedil inhibition of A23187-evoked acetylcholine release. (3) In the same experiment, we measured the effect of cetiedil alone and the effect of cetiedil in the presence of cyclosporin A (0.5–10 μM) added 3 min before. (4) Increasing cyclosporin A concentrations were applied on synaptosomes for 2 min prior to the addition of 20 μM cetiedil and acetylcholine release was then induced with 4 mM Ca^{2+} and 4 μM A23187. Results are the mean ± S.E.M. from 3–5 experiments. C: Cyclosporin A prevents MR16728 stimulation of A23187-evoked acetylcholine release. The same protocol was used with MR16728. Acetylcholine release was performed in the presence of 40 μM MR16728 or with 10 μM cyclosporin A. The same cyclosporin A concentration was then applied on synaptosomes 2 min before the addition of 40 μM MR16728 and acetylcholine release was triggered by Ca^{2+} and A23187 3 min later. Traces of a representative experiment are presented (5) and in the histogram (6), results are the mean ± S.E.M. from three independent experiments. * Significantly different from control values ($P < 0.02$).

solution 5 min before [^{3}H]cetiedil; the specific binding was determined as previously described.

3. Results

Acetylcholine release from *Torpedo* synaptosomes preincubated with various concentrations of cyclosporin A (0.05–4 μM) for 5 min was measured using the choline oxidase chemiluminescent procedure. For each sample, acetylcholine release was calibrated by internal acetylcholine standards and both areas under traces were measured; in each experiment the amount of acetylcholine release was expressed as a percentage of the control values (observed in the absence of drug). The amount of control acetylcholine release induced by 60 mM KCl was 43.4 ± 17.9 pmol (mean ± S.E.M. obtained in 5 separate experiments) representing 7.2 ± 1.9% of total acetylcholine content present in the synaptosomal preparations. The application of cyclosporin A on synaptosomes does not induce any significant basal release (data not shown). Acetylcholine release induced by 60 mM KCl in the presence of 4 mM Ca^{2+} was inhibited by cyclosporin A (Fig. 1, A1). This inhibition was a function of the concentration of cyclosporin A; half-inhibition was obtained at a concentration of cyclosporin A of 1 μM in the release solution (Fig. 1, A2). About 50% of acetylcholine release was resistant to the action of 4 μM cyclosporin A. A similar dose-response curve inhibition of cyclosporin A was obtained when acetylcholine was induced by 12 mM KCl (data not shown).

The increase in the concentration of Ca^{2+} in nerve terminals which triggers acetylcholine release can be obtained by adding a Ca^{2+} ionophore. A synaptosomal acetylcholine content of 16.2 ± 1.6% was released by 4 μM of the ionophore A23187 corresponding to 143 ± 63 pmol acetylcholine released (mean values ± S.E.M. from 5 separate preparations). Cyclosporin A (up to a concentration of 10 μM in the release solution) does not affect significantly A23187-induced acetylcholine release.

Cetiedil and its analogue MR16728 were previously characterized as substances which respectively inhibit and stimulate A23187-evoked acetylcholine release from *Torpedo* synaptosomes. In the present work, we observed the effect of pretreatment of synaptosomes with cyclosporin A on the effect of cetiedil and MR16728. The inhibitory effect of cetiedil (20 μM) on acetylcholine release induced by the addition of A23187 was observed in the presence of cyclosporin A. Cyclosporin A at various concentrations was added to the release solution containing synaptosomes; 2 min later, 20 μM cetiedil was added. Acetylcholine release was triggered after a 3-min incubation with cetiedil by adding Ca^{2+} (4 mM) and A23187 (4 μM). In the absence of cyclosporin A, the inhibition due to 20 μM cetiedil is almost total (Fig. 1, B3); the previous addition of cyclosporin A (0.5–10 μM) in the release solution prevents cetiedil from being active in inhibiting acetylcholine release. As shown in Fig. 1 (B4), 4 μM cyclosporin A allows the restoration of the control level of acetylcholine release (measured in the absence of drug).

A similar protocol was used to study the effect of cyclosporin A on the MR16728 stimulation of acetylcholine release in *Torpedo* synaptosomes. Cyclosporin A (10 μM) was added to the release solution; 2 min later, MR16728 (40 μM) was applied on synaptosomes. Acetylcholine release was triggered 3 min later with Ca^{2+} (4 mM) and A23187 (4 μM). MR16728 (40 μM) enhances acetylcholine release (around 140% of control); the addition of cyclosporin A (10 μM) prior to MR16728 abolishes the stimulatory effect of this cetiedil analogue (Fig. 1, C).

To investigate a possible role for extracellular Na^+ and Na^+ channels in the process of the stimulated acetylcholine release from *Torpedo* synaptosomes, we measured the tetrodotoxin sensitivity of KCl- and ionophore-induced acetylcholine release. Tetrodotoxin (1.5 μM, 5 min) inhibits slightly but not significantly (data not shown) both types of acetylcholine release. We observed also that the previous addition of tetrodotoxin does not modify the effects of cetiedil and MR16728 on both KCl- and A23187-induced acetylcholine release, and does not impair the reversal effects of cyclosporin A, leading to the conclusion that KCl- and A23187-induced acetylcholine releases are not significantly affected by the blockade of tetrodotoxin-sensitive Na^+ channels in *Torpedo* synaptosomes.

We examined the binding of [^{3}H]cetiedil on presynaptic membranes purified from *Torpedo* electric organ synaptosomes according the method of Morel et al. (1982). Non-specific [^{3}H]cetiedil binding was determined in the presence of 225 μM non-radioactive cetiedil and was 23–76% of total binding for concentrations of [^{3}H]cetiedil from 30 to 1100 nM. [^{3}H]Cetiedil binding is saturable (Fig. 2, A). A Scatchard analysis indicates a dissociation constant (K_D) of 181.9 ± 23.8 nM and a receptor density (B_{max}) of 44.1 ± 5.8 pmol/mg protein (Fig. 2, A).

We used the same protocol and evaluated [^{3}H]cetiedil binding for concentrations of [^{3}H]cetiedil of 100 and 500 nM. Various concentrations of MR16728 or cyclosporin A, between 2 and 50 μM, were added 5 min before cetiedil. MR16728 and cyclosporin A do not modify [^{3}H]cetiedil non-specific binding on presynaptic membranes. We also established that the solution containing cyclosporin A did not modify [^{3}H]cetiedil specific binding. Dixon representations (Fig. 2, B and C) show that MR16728 and cyclosporin A inhibit

[^{3}H]cetiedil binding on presynaptic membranes in a competitive way and allow to determine an inhibition constant (K_I) of 11.7 and 1.7 μM, respectively.

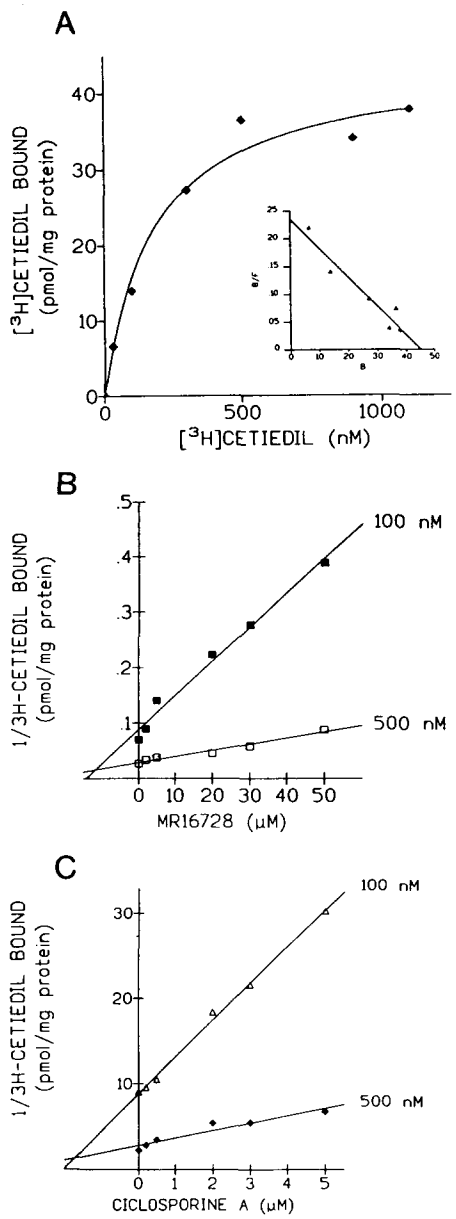

Fig. 2. A: Cetiedil binding on purified synaptosomal presynaptic membranes. Purified presynaptic membranes were incubated for 1 h on ice with increasing concentrations of [^{3}H]cetiedil; the specific radioactivity was 7 Ci/mmol. Bound [^{3}H]cetiedil was measured by filtration. Non-specific binding was measured in the presence of 225 μM non-radioactive cetiedil and was subtracted from total binding to obtain specific binding. Results are the mean of duplicate determinations from four independent experiments. Insert: Scatchard representation. B: Effect of MR16728 on [^{3}H]cetiedil binding. Various concentrations of MR16728 were added to presynaptic membranes 5 min before the addition of 100 nM or 500 nM [^{3}H]cetiedil. A Dixon analysis is presented. Each point is the mean of duplicate determinations from three experiments. C: Effect of cyclosporin A on [^{3}H]cetiedil binding. The same protocol was used with cyclosporin A instead of MR16728. Results, duplicate determinations from four independent experiments, are presented with a Dixon analysis.

4. Discussion

The immunosuppressive agent cyclosporin A was previously shown to inhibit the release of various substances due to a Ca^{2+} influx such as the release of insulin from pancreatic islets (Draznin et al., 1988) or the release of lactoferrin from polymorphonuclear leucocytes during the process of degranulation (Forrest et al., 1991), suggesting that cyclosporin A may be a useful tool in the dissection of the molecular release mechanism. Similarly, cyclosporin A inhibits in the same range of concentration the release of acetylcholine induced by KCl in *Torpedo* synaptosomes. Cyclosporin A affects in a specific way the entry of Ca^{2+} in synaptosomes since it is inactive in acetylcholine release induced with ionophore A23187. Therefore it may affect voltage-dependent Ca^{2+} entry through channels on synaptosomal presynaptic membranes. An interaction of cyclosporin A with Ca^{2+} channels was previously reported and the use of Ca^{2+} channel inhibitors such as diltiazem or verapamil induces an increase in cyclosporin A blood levels in transplant patients who receive immunosuppressive treatment (Leibbrandt and Day, 1992). In contrast, the step of acetylcholine release which takes place after the Ca^{2+} influx is not affected by cyclosporin A since this agent does not act on acetylcholine release induced by a Ca^{2+} ionophore.

Cyclosporin A also prevents cetiedil and MR16728 from being active in acetylcholine release induced by the ionophore A23187. A potential target of these agents is the mediatophore, a presynaptic membrane protein, since the effect of inhibition and stimulation of acetylcholine release due to cetiedil and MR16728, respectively, were also observed in acetylcholine-containing proteoliposomes in which this protein has been incorporated and which release acetylcholine in a Ca^{2+}-dependent way (Morot Gaudry-Talarmain et al., 1987; Moulian et al., 1993). We cannot exclude that the effect of cyclosporin A on *Torpedo* synaptosomes could be related to other presynaptic proteins associated with the mediatophore or involved in the release mechanism itself. Helekar et al. (1994) proposed that cyclophilin, a ubiquitous cytoplasmic protein which is blocked by cyclosporin A, may play a critical role in the maturation and function of homooligomeric receptors such as 5-HT_3 receptor and nicotinic α7 receptor. It could act as a peptidyl-prolyl *cis-trans* isomerase on the folding of these proteins. Mediatophore is composed of homooligomers of 15 kDa and contains in its peptidic sequence several prolines (Birman et al., 1990). This proteolipid could be an indirect target of cyclosporin A.

Moreover, the effects of cetiedil on morphologic changes of synaptosomal membranes observed by cryofracture after stimulation were previously studied in

our laboratory. Cetiedil blocks the rearrangement of large intramembrane particles which was shown to be associated with acetylcholine release regardless of the stimulation (Israël et al., 1987). Thus, the effects of cetiedil on acetylcholine release in synaptosomes may be related to its action on mediatophore conformation. Cyclophilin, which modifies homooligomer protein folding, could in this way prevent cetiedil and MR16728 from being active in the mediatophore.

We performed binding experiments with [^{3}H]cetiedil to study the interactions between cyclosporin A, cetiedil and MR16728. We measured its binding on purified synaptosomal presynaptic membranes (Fig. 2, A). We showed that radioactive cetiedil specifically binds to these membranes with a K_D of 180 nM and a B_{max} of 44.1 pmol/mg of protein. The competitive inhibitions of the binding of [^{3}H]cetiedil on synaptosomal presynaptic membranes obtained with MR16728 and cyclosporin A show that cetiedil, its analogue and cyclosporin A bind to a same site on these membranes. Cyclosporin A is 6 times more efficient than MR16728 in the inhibition of [^{3}H]cetiedil binding; we determined an inhibition constant (K_I) of 11.7 μM for MR16728 and 1.7 μM for cyclosporin A. The effect of cyclosporin A on cetiedil inhibition of acetylcholine release in *Torpedo* synaptosomes could be related to the inhibition of cetiedil binding by cyclosporin A.

In conclusion, in *Torpedo* synaptosomes, the most promising targets of cyclosporin A effects are voltage-sensitive Ca^{2+} channel entry and mediatophore or another presynaptic protein directly involved in acetylcholine release. These targets could be indirectly affected via cyclophilin. We plan to investigate if cyclophilin is present on *Torpedo* synaptosomes. In this case, isolated *Torpedo* nerve terminals could provide a useful tool for studying the action of cyclosporin A and one of its targets, cyclophilin, on the function of a presynaptic membrane protein (or other associated proteins) which controls acetylcholine release.

Acknowledgements

This work was supported by grants from the CNRS (Centre National de la Recherche Scientifique), DRET (Direction des Recherches, Etudes et Techniques; contract No. 92-175), Servier Pharmaceutics. N.M. was a Ph.D. student of the IFSBM (Institut de Formation Supérieure Bio-Médicale). The authors would like to thank Dr Maurice Israël for helpful discussions and Dr S. O'Regan for critically reading the manuscript, Dr Jean-Louis Morgat, Département d'Ingénierie des Protéines, Centre d'Etudes Nucléaires de Saclay, Gif-sur-Yvette, France, for synthesizing tritiated cetiedil, and Drs Max Robba, Jean-Charles Lancelot, Carmela Saturnino, Laboratoire de Chimie Thérapeutique, U.E.R. des Sciences Pharmaceutiques, Université de Caen, France, for synthesizing cetiedil and MR 16728.

References

Atkinson, K., J. Biggs, P. Darveniza, J. Boloand, A. Concannon and A. Dodds, 1984, Cyclosporin-associated central nervous system toxicity after renal transplantation, Transplantation 38, 34.

Birman, S., F.M. Meunier, B. Lesbats, J.P. Le Caër, J. Rossier and M. Israël, 1990, A 15 kDa proteolipid found in mediatophore preparations from *Torpedo* electric organ presents high sequence homology with the bovine chromaffin granule protonophore, FEBS Lett. 261, 303.

Draznin, B., S.A. Metz, K.E. Sussman and J.W. Leitner, 1988, Cyclosporin-induced inhibition of insulin release, Biochem. Pharmacol. 37, 3941.

Forrest, M.J., M.E. Jewell, G.C. Koo and N.H. Sigal, 1991, FK-506 and cyclosporin A: selective inhibition of calcium-ionophore-induced polymorphonuclear leukocyte degranulation, Biochem. Pharmacol. 42, 1221.

Helekar, S.A., D. Char, N. Shawn and J. Patrick, 1994, Prolyl isomerase requirement for the expression of functional homo-oligomeric ligand-gated ion channels, Neuron 12, 179.

Hohman, R.J. and T. Hultsch, 1990, Cyclosporin A: new insights for cell biologists and biochemists, New Biologist 2, 663.

Israël, M. and B. Lesbats, 1981, Chemiluminescent determination of acetylcholine and continuous detection of its release from *Torpedo* electric organ synapses and synaptosomes, Neurochem. Int. 3, 81.

Israël, M., R. Manaranche, Y. Morot Gaudry-Talarmain, B. Lesbats, T. Gulik-Krzywicki and J.C. Dedieu, 1987, Effect of cetiedil on acetylcholine release and intramembrane particles in cholinergic synaptosomes, Biol. Cell 61, 59.

Leibbrandt, D.M. and R.O. Day, 1992, Cyclosporin and calcium channel blockers: an exploitable drug interaction? Med. J. Aust. 157, 296.

Morel, N., M. Israël, R. Manaranche and P. Mastour-Frachon, 1977, Isolation of pure cholinergic nerve endings from electric organ, J. Cell Biol. 75, 43.

Morel, N., R. Manaranche, M. Israël and T. Gulik-Krzywicki, 1982, Isolation of a presynaptic membrane fraction from *Torpedo* cholinergic synaptosomes: evidence for a specific protein, J. Cell Biol. 93, 349.

Morot Gaudry-Talarmain, Y., M. Israël, B. Lesbats and N. Morel, 1987, Cetiedil, a drug that inhibits acetylcholine release in *Torpedo* electric organ, J. Neurochem. 49, 548.

Moulian, N., Y. Morot Gaudry-Talarmain and M. Israël, 1993, The effect of MR16728, a cetiedil analogue, on acetylcholine release in *Torpedo* synaptosomes, Eur. J. Pharmacol. 231, 407.

Moulian, N., Y. Morot Gaudry-Talarmain and M. Israël, 1994, Spontaneous acetylcholine from *Torpedo* synaptosomes: effect of cetiedil and its analogue MR16728, J. Neurochem. 62, 113.

Robba, M. and Y. Le Guen, 1967, Dérivés thiophéniques β-substitués. 3. Esters aminés des acides α-phényl-α-cyclopentyl et α-cyclohexyl-α-(thiényl)-3) acétiques, Chim. Thér. 2, 120.