Image Cover Sheet

CLASSIFICATION	SYSTEM NUMBER
UNCLASSIFIED	509355

TITLE

EFFECT OF RUTHENIUM RED ON VOLTAGE-SENSITIVE CA++ CHANNELS

System Number:

Patron Number:

Requester:

Notes:

DSIS Use only:

Deliver to:

OL 93-012

0022-3565/95/2732-0940$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

JPET 273:940–947, 1995

Vol. 273, No. 2
Printed in U.S.A.

Effect of Ruthenium Red on Voltage-Sensitive Ca^{++} Channels[1]

MURRAY G. HAMILTON[2] and PAUL M. LUNDY

Pharmacology and Therapeutics, Defence Research Establishment Suffield, P.O. Box 4000, Medicine Hat, Alberta, Canada T1A 8K6

Accepted for publication January 3, 1995

ABSTRACT

The organometallic dye, ruthenium red (RR) inhibited Ca^{++} influx, ω-conotoxin GVIA-sensitive Ca^{++} binding and Ca^{++}-dependent neurotransmitter release in a qualitatively similar manner in rat and chicken synaptosomes and in mammalian neuromuscular preparations, but had no effect on Ca^{++}-dependent processes mediated by the dihydropyridine-sensitive Ca^{++} channel. These effects are specific for the RR complex, as $RuCl_3$ affected neither Ca^{++} influx, ω-conotoxin GVIA binding nor neurotransmitter release, but did, however, in contrast to RR, displace [^{3}H]nitrendipine from synaptosomes. RR, in a manner similar to ω-conotoxin MVIIC and ω-agatoxin IVA (AgaIVA), also effectively inhibited the response of the rat diaphragm to nerve stimulation and blocked AgaIVA-sensitive Ca^{++} influx in the rat brain, suggesting a significant interaction at the P-type voltage-sensitive Ca^{++} channel. These effects of RR suggest that this amino complex affects both the N and the P domain of the Ca^{++} channel in the chicken brain and both the N- and P-type Ca^{++} channel which is intimately coupled to the Ca^{++} influx and neurotransmitter release in rat synaptosomes. Its ability to block all of the Ca^{++} influx in mammalian brain preparations and to inhibit completely the nerve-stimulated rat neuromuscular junction certainly indicates a significant action at the P-type voltage-sensitive Ca^{++} channel, similar to ω-conotoxin MVIIC or AgaIVA. RR should prove to be a valuable synthetic, inexpensive tool with which to probe the neuropharmacology of the mammalian neurotransmitter-linked voltage-sensitive Ca^{++} channels.

VSCCs govern the inward flux of Ca^{++} in presynaptic terminals after neuronal depolarization. It is this transient rise in intraterminal Ca^{++} concentrations which initiates a number of important events, particularly the release of NT.

RR is an organometallic dye which has a number of interesting effects on a variety of neuronal systems. Many of its effects have been shown to be a result of an ability to interfere with Ca^{++}-dependent processes. For example, i.p. injection of RR causes a flaccid paralysis which is reversed by elevation of Ca^{++} levels (Tapia, 1985; Ugalde-Garcia and Tapia, 1991) and intracisternal injection of RR causes convulsions which mimic the effects produced by the Ca^{++} chelator EDTA and are blocked by administration of $CaCl_2$ (Tapia and Arias, 1981; Tapia, 1985; Arias and Tapia, 1986). Furthermore, RR has been shown to block γ-[^{3}H]aminobutyric acid release and $^{45}Ca^{++}$ uptake at similar concentrations, to inhibit the binding of Ca^{++} to synaptic membranes (Kamino *et al.*, 1976; Goddard and Robinson, 1976; Tapia, 1985) and to decrease the cooperative nature of Ca^{++} association with presynaptic nerve endings (Kamino *et al.*, 1976). Several investigations have shown that RR inhibits the depolarization-induced uptake of Ca^{++} in synaptosomes and that the blockade of Ca^{++} transport is functionally coupled to inhibition of NT release (Meza-Ruiz and Tapia, 1978; Tapia, 1985). Moreover, RR, by interfering with undefined Ca^{++} channels, selectively antagonizes capsaicin-induced Ca^{++} uptake and the subsequent release of various neuropeptides (Maggi *et al.*, 1988; Franco-Cereceda *et al.*, 1989) and NTs (Tapia and Arias, 1981; Tapia *et al.*, 1985; Arias and Tapia, 1986; Sitges, 1989; Ugalde-Garcia and Tapia, 1991). These results strongly suggest that RR inhibits the interaction of Ca^{++} with a VSCC.

Several types of VSCCs have been identified that affect at least certain parts of the neuron. The L-type VSCC governs Ca^{++} influx, but the greatest portion of this flux does not appear to regulate NT release at most central synapses (Nachshen and Blaustein, 1980; Rampe *et al.*, 1984; Skattebøl and Triggle, 1987a,b; Miller, 1987). The N-type VSCC regulates a portion (up to 40%, depending on the study) of the depolarization-evoked NT release from mammalian synaptosomes (Reynolds *et al.*, 1986; Woodward *et al.*, 1988; Lundy *et al.*, 1991) and brain slices (Dooley *et al.*, 1988; Herdon and Nahorski, 1989) and is predominantly or exclusively respon-

Received for publication April 18, 1994.

[1] In conducting this research the authors adhered to the "Guide to the Care and Use of Experimental Animals" and "The Ethics of Animal Experimentation" published by the Canadian Council on Animal Care.

[2] Present Address: Département de Pharmacologie, Unité de Neurotoxicologie, Centre de Recherches du Service de Santé des Armées, 24, av. des Maquis du Grésivaudan, B.P. 87, 38702 La Tronche Cedex, France.

ABBREVIATIONS: VSCC, voltage-sensitive Ca^{++} channel; NT, neurotransmitter; RR, ruthenium red; DHP, dihydropyridine; ω-CgTx, ω-conotoxin GVIA; ω-CmTx, ω-conotoxin MVIIC; AgaIVA, ω-agatoxin IVA; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PSS, physiological salt solution; NRB, normal resting buffer; DA, dopamine; ΔK^+, (depolarized-resting) K^+-stimulated Ca^{++} influx; CI, confidence intervals.

sible for the Ca^{++} influx which governs NT release from mammalian autonomic nerves (Lundy and Frew, 1988, 1993, 1994; Keith *et al.*, 1989; Plummer *et al.*, 1989; De Luca *et al.*, 1990). There is an additional significant portion of Ca^{++} influx in mammalian central nervous system preparations which has been shown both pharmacologically (Woodward *et al.*, 1988; Suszkiw *et al.*, 1989; Lundy *et al.*, 1991; Mangano *et al.*, 1991) and electrophysiologically (Regan *et al.*, 1991; Pfrieger *et al.*, 1992; Allen *et al.*, 1993) to be insensitive to both L- and N-type VSCC antagonists. This DHP, ω-CgTx-insensitive Ca^{++} influx may represent Ca^{++} entry through the P channel (Llinás *et al.*, 1989; Llinás *et al.*, 1992; Mintz *et al.*, 1992).

It is now accepted that the depolarization-dependent Ca^{++} influx leading to NT release occurs through either VSCCs of the N-type or an additional type which is DHP- and ω-CgTx-insensitive, and is probably either the P VSCC or the recently described ω-CmTx-sensitive Q VSCC (Wheeler *et al.*, 1994). With the exception of its effect on L VSCC binding (Hansford, 1987; Massieu and Tapia, 1988), the effects of RR on Ca^{++} influx specifically associated with a NT coupled VSCC and on the binding of ligands to identified VSCCs which are known to play a role in NT release (*i.e.*, N or P) have not been investigated systematically. The present work was designed to examine the effects of RR on Ca^{++} influx by using tissues with pharmacologically defined VSCCs. These tissues include those rich in ω-CgTx- and AgaIVA-sensitive channels coupled to NT release such as the VSCCs in chicken brain synaptosomes (N-type or the NP-type) (Lundy *et al.*, 1994), and the P-type VSCCs defined in the rat neuromuscular junction (Uchitel *et al.*, 1992) or in the rat brain (Mintz *et al.*, 1992). We also report the effect of RR on the specific binding of N and L channel ligands to rat and chicken synaptosomes.

Methods

Preparation of synaptosomes for binding studies. Synaptosomes were derived from freshly dissected cerebral hemispheres of male Sprague-Dawley rats (approximately 200 g, Charles River, Montreal, Quebec, Canada) or forebrains of adult Leghorn chickens (approximately 2 kg, La Poulet, Medicine Hat, Alberta, Canada). The brain tissue was homogenized in 10 volumes (w/v) of ice-cold 0.32 M sucrose containing 0.5 mM EDTA in a Teflon/glass homogenizer by using 10 strokes at 750 rpm. The homogenate was centrifuged at $1000 \times g$ for 10 min and the supernatant was pelleted at $12{,}500 \times g$ for 25 min, all at 4°C. This pellet (P_2) was washed with ice-cold resting buffer (HEPES-based Krebs' solution, containing, in millimolar: NaCl, 132; KCl, 5; glucose, 10; and HEPES, 10; at pH 7.4). In the case of [^{3}H]nitrendipine, 1 mM $CaCl_2$ was added to the buffer and in the case of ω-[^{125}I]conotoxin, $CaCl_2$ was omitted from the binding solution to minimize interference with N channel binding (Knaus *et al.*, 1987; Ballesta *et al.*, 1989). After determination of protein concentration (Coomassie blue reagent, Pierce Chemicals, Rockford, IL), synaptosomes were diluted with 50 mM Tris · HCl, pH 7.4, containing 150 mM NaCl and 2 mM $CaCl_2$ ([^{3}H]nitrendipine binding) or 50 mM Tris · HCl (ω-[^{125}I]conotoxin binding).

Binding studies. Rat or chicken synaptosomes, prepared as described above, were suspended in the appropriate buffer at a concentration of 40 to 80 μg/ml (representing 2 to 4 μg of protein per tube for ω-CgTx) or 400 to 800 μg/ml (nitrendipine) and were used in the binding assay. ω-[^{125}I]CgTx (specific activity, 2200 Ci/mmol, New England Nuclear, Boston, MA), obtained as a lyophilized powder, was dissolved in distilled H_2O. Dissociation curves were obtained by incubating unlabeled RR, $RuCl_3$ or $CdCl_2$ at 37°C for 60 min in the presence of 20 to 30 pmol of ω-[^{125}I]CgTx. The reaction was terminated by using ice-cold buffer containing 0.2% bovine serum albumin and 200 mM NaCl. Membranes were collected on GFC filters by using a Brandel Harvester, washed twice with buffer and counted in a scintillation counter as above. Nonspecific binding was determined in the presence of 10^{-7} M unlabeled ω-conotoxin.

[^{3}H]-Nitrendipine, obtained from New England Nuclear (70.7 Ci/mmol) was dissolved in distilled H_2O. The ligand (0.8–1.5 nmol) was incubated as above in Tris · HCl containing 150 mM NaCl and 2 mM $CaCl_2$ with 20 to 40 μg of the synaptosomal dilutions for 60 min at 37°C. The reaction was terminated by using ice-cold incubation buffer containing 0.2% bovine serum albumin. Protein was collected on GFC glass-fiber filters by using a Brandel cell harvester (Brandel, Gaithersburg, MD), washed twice with buffer and counted as above. Nonspecific binding was determined in the presence of 10^{-5} M unlabeled nifedipine.

Curves were fitted to the binding data by using nonlinear least-squares fitting techniques (GraphPad, San Diego, CA). Statistical analyses (analysis of variance and multiple comparisons) were performed by using RS/1 (Bolt, Beranek and Newman, Boston, MA).

Preparation of synaptosomes for Ca^{++} influx studies. Cortical tissue from rats or forebrain from chickens was homogenized in 0.32 M sucrose by using a motor-driven Teflon-glass homogenizer. The homogenate was centrifuged at 4°C, $1000 \times g$ for 10 min. The supernatant was decanted and centrifuged at $12{,}400 \times g$ for 25 min and the pellet was resuspended in HEPES-buffered PSS containing, in millimolar: choline chloride, 132; KCl, 5; $MgCl_2$, 1.3; $CaCl_2$, 1.5; NaH_2PO_4, 1.2; D-glucose, 10; and HEPES, 20; adjusted to pH 7.4 with Tris base. The protein concentration was adjusted to 1.0 to 1.5 mg/ml with PSS.

Ca^{++} influx. Ca^{++} influx was carried out essentially as described previously (Lundy *et al.*, 1989). Briefly, various concentrations of ω-CgTx or RR were preincubated together with the synaptosomes for 15 min (5 mM K^+) at 30°C. At the end of the incubation period, a 100-μl aliquot of this synaptosomal suspension was injected into PSS or depolarizing PSS (PSS in which 20 mM K^+, final concentration, was substituted iso-osmotically for choline) containing 0.5 to 1 μCi of $^{45}CaCl_2$ (New England Nuclear). The influx was allowed to proceed for various time periods up to 30 sec, when the influx was stopped by the rapid dilution of the suspension with 4 ml of ice-cold quench buffer (Ca^{++}-free choline resting buffer containing 4 mM ethylene glycol bis(β-aminoethyl ether)-N,N′-tetraacetic acid. The synaptosomes in the quench buffer were filtered rapidly under vacuum on 0.45-μm Gelman GA6-S filters on a Hoeffer filtration apparatus (Hoeffer Scientific, San Francisco, CA) and washed twice with this same buffer. The filters were allowed to dry and the radioactivity trapped by the filters was determined by liquid scintillation spectroscopy.

NT release. Synaptosomes were prepared from freshly dissected forebrains of either chickens or rats as described under *Binding*. The P_2 pellet was carefully resuspended in ice-cold NRB containing, in millimolar: glucose, 10; NaCl, 125; KCl, 5; HEPES, 10; ascorbic acid, 10; and pargyline, 0.01; at pH 7.4 (Tris). This suspension was centrifuged again at $20{,}000 \times g$ for 15 min and the resulting pellet was used for the NT release experiments.

The pellet was resuspended in NRB at a concentration of approximately 1 mg of protein per ml. Two milliliters of this suspension was incubated at 37°C with 20 nM [^{3}H]DA (specific activity, 18.6 Ci/mmol; New England Nuclear, Mississauga, Ontario, Canada) for 20 min. Subsequently, 300 μl of these [^{3}H]DA-labeled synaptosomes were loaded into each channel of a 6-well superfusion apparatus (Brandel) and superfused with NRB at 37°C and a flow rate of 0.75 ml/min. After a 30-min equilibration period in the superfusion chambers, fractions were collected every 2 min. NT release was effected by substituting NRB with buffer in which K^+ (25 mM final concentration) had been iso-osmotically substituted for Na^+ (normal substituted buffer). Drug additions were made to the NRB during equilibration and to both the NRB and normal substituted buffer during

fraction collection. The superfusates and the filters (Whatman GF/C filter discs) containing the ^3H remaining in the synaptosomes were counted by liquid scintillation spectroscopy. Fractional release of NT was calculated as the radioactivity of the fraction collected minus the total remaining radioactivity in the synaptosomes. That is:

$$F_R = R_T - \sum_{1}^{i-1} R_{Fi}$$

where F_R is the fractional release of [^{3}H], R_T is the total radioactivity of the synaptosomal preparation at the beginning of the experiment (*i.e.*, the radioactivity remaining in the pellet and the sum of all fractional releases) and R_{Fi} is radioactivity of the ithfraction.

Preliminary experiments showed that the depolarization-evoked NT release was Ca^{++} dependent (more than 95% decrease in stimulated release in Ca^{++}-free buffer) and that synaptosomes did not accumulate sufficient NT to allow two stimulations with K^+. Therefore, control and drug-treated release experiments were run in parallel and compared to the evoked release from a single 25 mM K^+ pulse.

Phrenic nerve hemi-diaphragm preparation. The left hemidiaphragm and attached phrenic nerve was carefully dissected from a male Sprague-Dawley rat after decapitation and mounted in an organ bath containing Krebs' solution at 37°C and oxygenated with 95% O_2 in CO_2. The nerve was stimulated by using a ring electrode with square-wave biphasic pulses at 0.2 Hz, 1 msec duration and supramaximal voltage. The twitches were recorded *via* an isometric transducer (Harvard Apparatus, Cambridge, MA) connected to a Rikadenki strip chart recorder. The effect of RR and ω-CgTx on nerve-mediated twitch height was determined in the presence of a threshold concentration of *d*-tubocurare (0.5–0.75 μM) to eliminate the large excess of postsynaptic receptors (Ginsberg and Hirst, 1972).

Chemicals. [^{3}H]Nitrendipine, ω-[^{125}]CgTx, [^{3}H]DA and $^{45}CaCl_2$ were obtained from New England Nuclear. ω-CgTx and ω-CmTx were from Bachem (Torrance, CA), the AgaIVA from Peptides International (Louisville, KY) and the remaining chemicals were from Sigma Chemical Co. (St Louis, MO).

Results

RR decreased the K^+ evoked (ΔK^+) influx of Ca^{++} in both chicken and rat synaptosomes (fig. 1, A and B, respectively). There was no effect on basal Ca^{++} uptake. Similarly, the nonspecific inorganic Ca^{++} channel inhibitor Cd^{++}, blocked over 90% of Ca^{++} influx in both tissues (results not shown). In the chicken brain, 1 μM ω-CgTx blocked 97% of the ΔK^+ (*i.e.*, stimulated-resting Ca^{++} influx) evoked Ca^{++} accumulation. However, unlike the effects of RR and the divalent cation Cd^{++}, $RuCl_3$ did not affect Ca^{++} translocation in either preparation. Calculations of the ED_{50} of RR for the inhibition of Ca^{++} translocation in chicken and rat synaptosomes yielded mean values of 2.5×10^{-4} M (95% CI: 0.9–4.3 $\times 10^{-4}$ M) and 6.9×10^{-5} M (95% CI: 6.3–8.3 $\times 10^{-5}$ M), respectively.

The effect of RR, $RuCl_3$ and $CdCl_2$ were analyzed with respect to their abilities to displace ω-[^{125}I]CgTx and [^{3}H]nitrendipine from rat and chicken synaptosomes. In concert with the results obtained with Ca^{++} influx studies, both RR and $CdCl_2$ inhibited the specific binding of ω-CgTx to both rat (fig. 2B) and chicken (fig. 2A) synaptosomes. The calculated IC_{50} values from the displacement curves were 2×10^{-6} (95% CI: 1.9–7.5 $\times 10^{-6}$ M) and 6×10^{-5} M (95% CI: 2.5–13 $\times 10^{-6}$ M) for RR and 1.5×10^{-5} (95% CI: 0.5–4 $\times 10^{-5}$ M) and 4×10^{-5} M (95% CI: 0.8–20 $\times 10^{-5}$ M) for $CdCl_2$ in rat and chicken brain synaptosomes, respectively. A similar

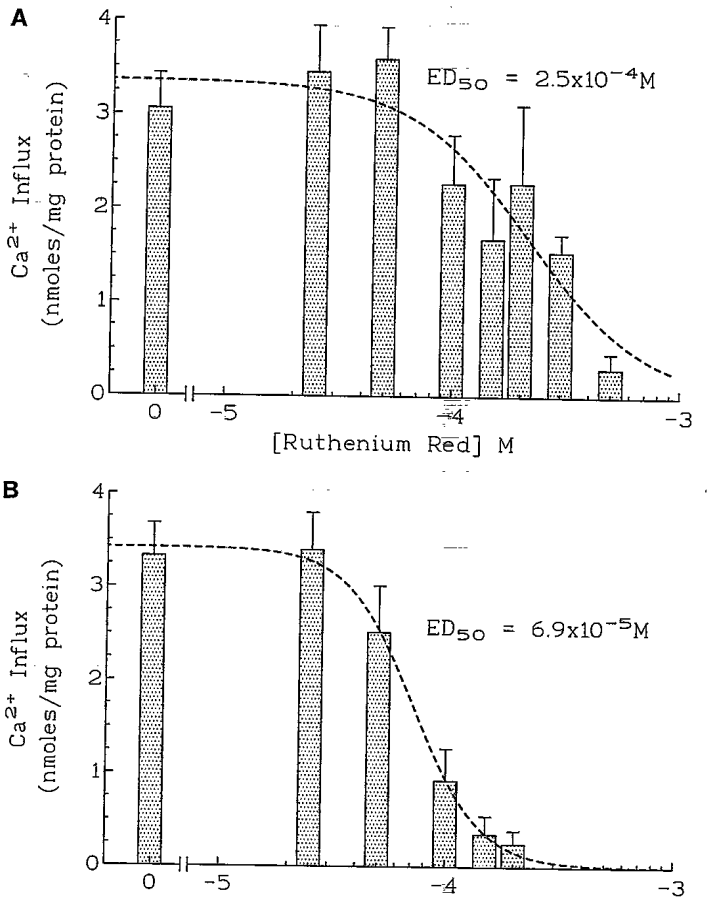

Fig. 1. Effect of RR on Ca^{++} influx in synaptosomes from chicken forebrain (A) or rat cortex (B). RR was added to chicken (A) or rat (B) synaptosomes for 15 min before depolarization in 25 mM K^+ (final concentration) PSS. The K^+-stimulated Ca^{++} influx (depolarized-resting $^{45}Ca^{++}$ accumulation; ΔK^+) was inhibited in a dose-dependent manner in both preparations. The ED_{50} values calculated after fitting the data by nonlinear least-squares analysis, show that RR is about 4 times more potent in rat brain preparations compared to chicken brain. Values are means of four experiments in triplicate ± S.E.

value could not be calculated for $RuCl_3$ because it had no significant effect on the specific binding of ω-[^{125}I]CgTx to synaptosomal membranes. In contrast to the results obtained with the specific binding of ω-[^{125}I]CgTx, RR did not affect the binding of the L-type VSCC ligand nitrendipine to either rat or chicken synaptosomes (fig. 3). $RuCl_3$ and $CdCl_2$, on the other hand, were very effective at displacing specifically bound [^{3}H]nitrendipine from the synaptosomes (table 1), indicating that the effect of RR is targeted primarily at the VSCC labeled by ω-[^{125}I]CgTx as opposed to the L channel.

The incubation of 20 nM [^{3}H]DA with synaptosomes isolated from the forebrains of chickens or rat striata resulted in a significant accumulation of ^{3}H-label. Most of this label was incorporated into the Ca^{++}-dependent releasable NT pool of the synaptosomes as evidenced by the lack of any K^+ stimulation of ^{3}H-overflow when external Ca^{++} concentrations were reduced to zero (data not shown). Both chicken (fig. 4A) and rat synaptosomes (fig. 4B) respond similarly to an increase in external K^+ (20 mM) with about a 3-fold increase in ^{3}H-overflow. The response to ω-CgTx as reported by others (Suszkiw *et al.*, 1987; Lundy *et al.*, 1991) is quantitatively quite different in the two species: 100 nM ω-CgTx inhibited

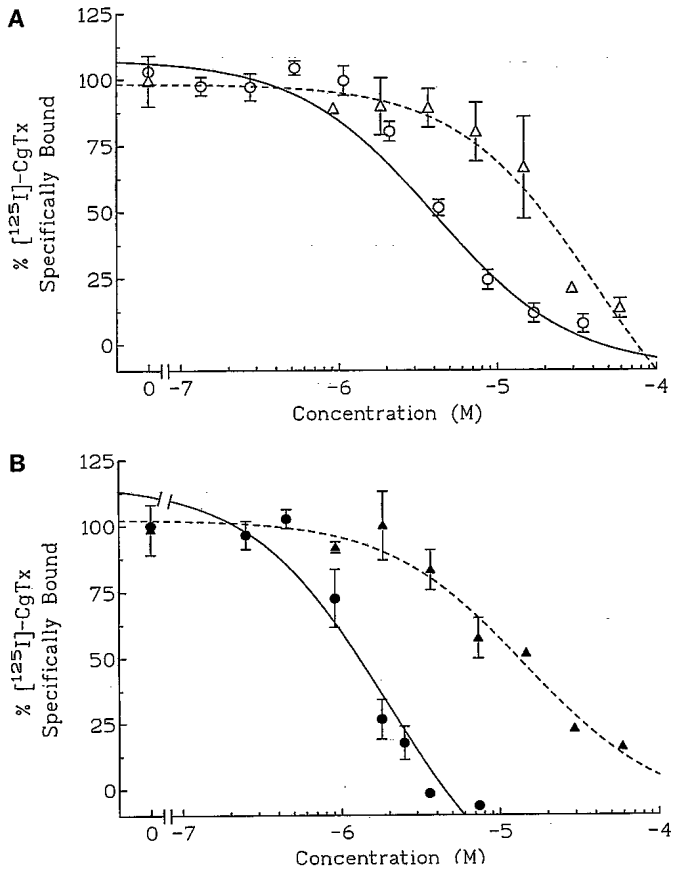

Fig. 2. Displacement of ω-CgTx binding by RR (●, ○) and $CdCl_2$ (▲, △) in chicken (A) and rat (B) synaptosomes. RR, $CdCl_2$ and $RuCl_3$ were incubated with 20 to 30 pmol of ω-[^{125}I]CgTx and synaptosomes. Both RR and $CdCl_2$ displaced specifically bound ω-CgTx with IC_{50} values for RR of 2 and 6 μM and for $CdCl_2$ of 15 and 40 μM in rat and chicken preparations, respectively. $RuCl_3$ did not affect ω-CgTx binding to either rat or chicken preparations at concentrations up to 100 μM (data not shown, see table 1). Values are means ± S.E. of at least two separate experiments in triplicate.

completely K^+ evoked [^{3}H]DA release in chicken synaptosomes, but at this low concentration had no effect on [^{3}H]DA release in rat synaptosomes (fig. 4). The response of these preparations to RR, on the other hand, was very similar both qualitatively and quantitatively. Concentrations of RR near the IC_{50} for the displacement of ω-[^{125}I]CgTx binding caused a significant depression of [^{3}H] overflow in both rat (fig. 4B) and chicken preparations (fig. 4A) and maximally effective concentrations of RR for ω-[^{125}I]CgTx displacement abolished completely K^+-stimulated [^{3}H]DA release (fig. 4A).

It is thought that the Ca^{++} channels coupled to NT release at the neuromuscular junction are the same P-type VSCC that have been described in brain tissue (Uchitel *et al.*, 1992). Therefore, known inhibitors of the P-type Ca^{++} channel were compared with the effects of RR on the phrenic nerve-diaphragm preparation. In this preparation, RR inhibited completely the nerve controlled twitch of the diaphragm in a dose-related manner with an IC_{50} very close to that which was found to inhibit the binding of ω-CgTx to synaptosomes and which also inhibited NT release (fig. 5E). ω-CgTx, on the other hand, had very little effect even at near maximally effective concentrations of 1 μM (fig. 5A). This finding agrees very well with previous results that also have failed to identify N-type VSCCs in this preparation by using ω-CgTx (Uchitel *et al.*, 1992). RR (fig. 5E) inhibited the response at the neuromuscular junction in a manner which was qualitatively similar to the actions of the P channel blocker AgaIVA (100 nM, fig. 5D) and the N, P and Q channel blocker, ω-CmTx (500 nM, fig. 5C). Although ω-CmTx has additional N channel blocking properties (Hillyard *et al.*, 1992), these are not likely to contribute to the actions of this peptide on the rat nerve-diaphragm preparation because the N-type Ca^{++} channel blocker ω-CgTx, as mentioned above, had no significant activity on this preparation.

Discussion

The rise in presynaptic intraterminal free Ca^{++} concentration initiates the biochemical events required for the release of NTs. The voltage-dependent influx of Ca^{++} which produces this rise occurs through at least two biophysically and pharmacologically distinct VSCCs (N and P) and a third with a debatable role (L-type). There are as well two other recently described subtypes, namely O and Q channels with, as yet, undefined roles in Ca^{++} gated neurotransmission. The first three VSCCs are specifically inhibited by different pharmacological agents, and are termed L, N and P (Saccomano and Ganong, 1991; Bertolino and Llinás, 1992). There is still controversy over which of these channels governs the majority of the depolarization-induced Ca^{++} influx that controls and initiates NT release in mammalian brain preparations. Several studies have shown that ω-CgTx-sensitive channels (*i.e.*, N channels) partially, but significantly, reduce Ca^{++} influx and the release of a number of NTs from mammalian brain preparations (Reynolds *et al.*, 1986; Dooley *et al.*, 1988; Herdon and Nahorski, 1989; Lundy *et al.*, 1991; Pullar and Findlay, 1992), whereas it is generally accepted that L-type VSCC inhibitors play a relatively minor role, usually in specific defined neuronal types and often only at physiologically high concentrations (Miller, 1987). Recent evidence suggests strongly that the majority of the ω-CgTx, DHP-insensitive Ca^{++} channels in mammalian brain which control Ca^{++} translocation and NT release are P-type (Mintz *et al.*, 1992; De Feo *et al.*, 1993; Turner *et al.*, 1993; Geer *et al.*, 1993). As well as the N, P and L series of VSCCs, two additional Ca^{++} channels, O and Q, have very recently been described. The Q channel is inhibited by micromolar concentrations of ω-CmTx (Wheeler *et al.*, 1994), and this same conotoxin also binds with high affinity to the O channel.

RR has been shown to inhibit Ca^{++} uptake and NT release in a variety of mammalian preparations (Tapia and Meza-Ruíz, 1977; Tapia, 1985; Maggi *et al.*, 1988; Wood *et al.*, 1988). Although these effects of RR are well defined, little information is available on the Ca^{++} channel(s) responsible for its effects. However, it has been shown previously that RR did not interfere with L channel binding (Massieu and Tapia, 1988), nor did it affect the rise in intracellular free Ca^{++} mediated by L-type channels in cardiac myocytes (Hansford, 1987). The results of the present study show clearly that RR specifically antagonizes the ω-CgTx-sensitive VSCC as well as an ω-CgTx, DHP-insensitive channel and support the findings of Massieu and Tapia (1988) in that RR does not displace the L-type VSCC ligand nitrendipine.

It was initially tempting to ascribe the inhibitory actions of

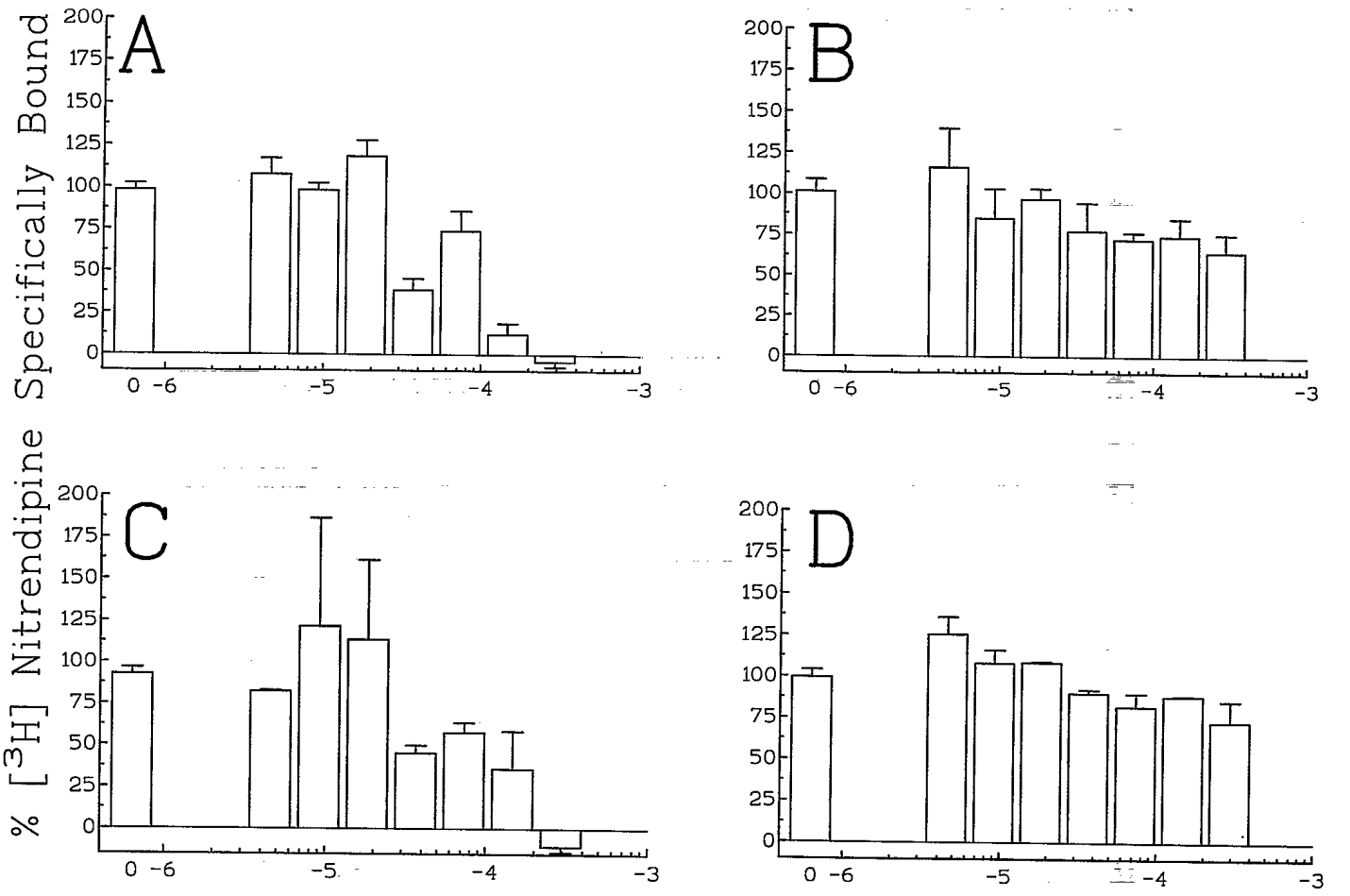

Fig. 3. Effect of RR and $RuCl_3$ on [^{3}H]nitrendipine binding to rat (C and D) and chicken (A and B) synaptosomes. Various concentrations of RR, $RuCl_3$ and $CdCl_2$ were incubated with [^{3}H]nitrendipine and chicken (upper panels) and rat (lower panels) synaptosomes as described under "Methods." Both $RuCl_3$ (A and C) and $CdCl_2$ (not shown) were effective in displacing the DHP from its binding site. RR, on the other hand, at concentrations 10 to 50 times those that displace ω-CgTx from its binding sites had no effect on [^{3}H]nitrendipine binding (B, chicken; D, rat). Results are means ± S.E. of two experiments in triplicate. [Note that at high concentrations $RuCl_3$ displaced more [^{3}H]nitrendipine than that used to define nonspecific binding (10^{-5} M). The same concentrations of RR, however, did not.]

TABLE 1

Effect of $RuCl_3$ and $CdCl_2$ on [^{3}H]Nitrendipine and ω-[^{125}I]conotoxin binding to rat and chicken synaptosomes

Values are mean percentage of specifically bound ligand ± S.E.M. from four experiments.

	[^{3}H]Nitrendipine (% specifically bound in presence of 500 μM competing ion)		ω-[^{125}I]Conotoxin (% specifically bound in presence of 30 μM competing ion)	
	Rat	Chicken	Rat	Chicken
$RuCl_3$	68.22 (20.9)	18.8 (12)	134.8 (4.6)	163.5 (36.5)
$CdCl_2$	43.3 (5.3)	13.1 (6.5)	18.66 (2.6)	16.5 (2.7)

RR to a molecular screening of the VSCC by Ru^{+++}, such as might be invoked for the actions of Cd^{++} or La^{+++} on VSCCs (Nachshen and Blaustein, 1980). However, two facts argue against this interpretation. First, $RuCl_3$ was ineffective in displacing ω-[^{125}I]CgTx binding from either rat or chicken synaptosomes and did not affect Ca^{++} influx. These results suggest that RR blockade of ω-CgTx-sensitive N channels is a result of the amino complex rather than the trinuclear chloro complexes $[Ru_3O_2Cl_6(H_2O)_6]$ found in aqueous solutions of $RuCl_3{\cdot}H_2O$. Thus, the specific binding of the amino complex of RR to the molecular structure of the N-type channel may be responsible for its activity, but other octahedral amino complexes of transition metals will need to be examined to understand this effect. Second, small concentrations of RR actually increase the apparent binding of ω-[^{125}I]CgTx to both rat and chicken synaptosomes, suggesting a more complex interaction between this organic ruthenium compound and the N-type VSCC than simple electrostatic screening. What is clear is that it is unlikely that ionic Ru^{+++} causes the inhibitory effect of RR on Ca^{++} influx, ω-CgTx binding or [^{3}H]DA release. There are several other oxidation states available to ruthenium and the possibility that one of these may have significant effects on VSCCs has not been ruled out by the present investigation.

The present results strongly support the conclusion that the various biological effects of RR are due to inhibition of VSCCs and point to which channels are affected. The ability of RR to inhibit the binding of ω-CgTx and to inhibit Ca^{++} influx and NT release in chicken brain initially led us to conclude that RR was a relatively specific inhibitor of N channels. However, several recent studies suggest that the N designation of the VSCC in the chicken brain may not be entirely accurate. It appears that this Ca^{++} channel shares

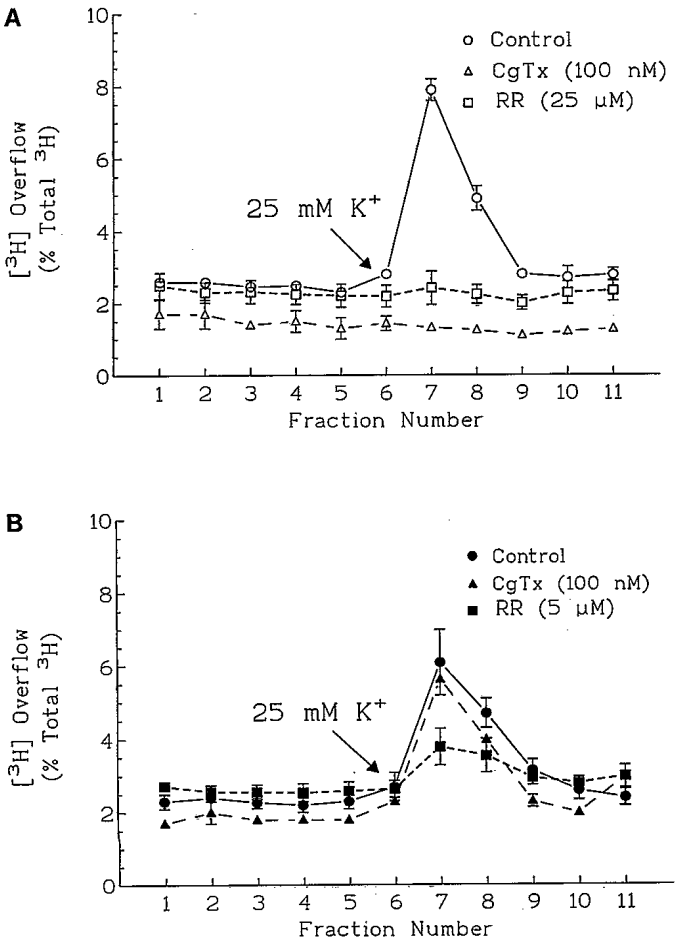

Fig. 4. Inhibition of [^{3}H]DA release by RR in chicken forebrain (A) and rat striatal (B) synaptosomes. RR, at concentrations in the low micromolar range, blocked the K^+-stimulated overflow of ^{3}H from synaptosomes of both species that had been incubated previously with radiolabeled DA. As with the binding and Ca^{++} influx, the RR was slightly more potent in rat preparations than chicken. ω-CgTx, at 100 nM, was as effective as RR in chicken synaptosomes in preventing [^{3}H]DA release, but ineffective at that concentration in rat striatal synaptosomes. Results are means ± S.E. from a single experiment in either species performed in duplicate. The experiment was repeated 3 times in each species with similar results.

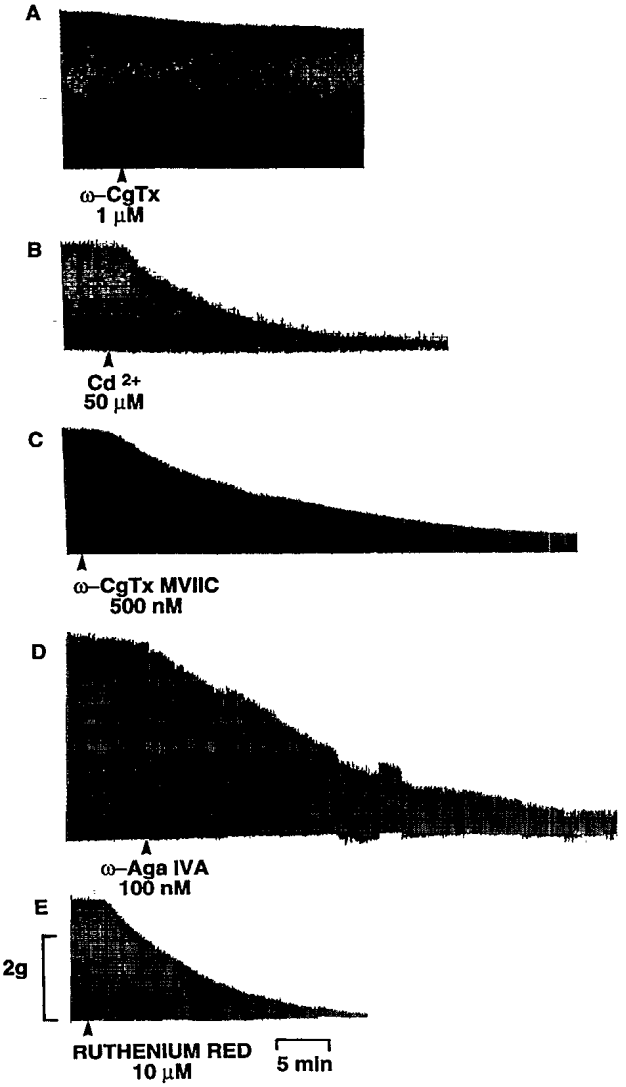

Fig. 5. Effect of RR on the nerve-stimulated rat diaphragm. The figure shows the effect of a single dose of 10 μM RR on the twitch height of the rat diaphragm elicited by electrical stimulation of the phrenic nerve. Several other compounds also were tested on this preparation of P channel-mediated neurotransmission. Cd^{++}, as expected (B), blocked the response of the diaphragm to nerve stimulation. The P channel blocker, ω-AgaIVA (100 nM, D) and the mixed N/P channel blocker ω-CmTx MVIIC (500 nM, C) had effects that were qualitatively similar to RR in inhibiting the twitch response to nerve stimulation. ω-CgTx, on the other hand, had no effect on this preparation at concentrations up to 1 μM (A).

characteristics of both N- and P-type VSCCs (Bowman *et al.*, 1993) and may represent a hybrid channel more properly designated as an NP VSCC (Lundy *et al.*, 1994). The fact that the chicken brain VSCC is sensitive to RR is not unexpected inasmuch as other studies carried out on P channels of the neuromuscular junction and the N channel of the peripheral autonomic nervous system are both inhibited by RR. Further evidence attesting to the effect of RR on N channels comes from its ability to inhibit ω-CgTx binding. It also has been reported in mammalian brain synaptosomes that the N-type VSCC accounts for up to 40% of the Ca^{++} influx (Reynolds *et al.*, 1986; Suszkiw *et al.*, 1989; Lundy *et al.*, 1991; Mangano *et al.*, 1991) and up to about 50% of the NT release (Reynolds *et al.*, 1986; Dooley *et al.*, 1988; Woodward *et al.*, 1988). These values are dependent on a variety of factors, the most important of which may be the depolarizing stimulus. In the present study, RR blocked K^+-stimulated Ca^{++} influx and NT release completely. These findings indicate that it blocked not only that proportion of the influx and release that is dependent on the N channel, but also the residual portion which is not subserved by the N channel. This ability of RR to inhibit the residual ω-CgTx insensitive portion of these parameters strongly implies that RR has P channel blocking activity as well. This inference is strengthened by recent evidence which shows that the majority of the ω-CgTx, DHP insensitive Ca^{++} influx and NT release in rat brain is modulated by the P type channel because it is antagonized by P channel blockers (Mintz *et al.*, 1992; De Feo *et al.*, 1993; Takahashi and Momlyama, 1993; Geer *et al.*, 1993; Lundy *et al.*, 1994). In addition, RR caused a dose-dependent inhibition of the rat diaphragm preparation in which nerve-stimulated acetylcholine release appears to be subserved by P-

type VSCCs (Uchitel *et al.*, 1992; Hillyard *et al.*, 1992; present study, fig. 5D) rather than N- or L-type VSCCs (Wessler *et al.*, 1990). The possibility that RR is affecting a Q channel cannot be excluded inasmuch as it mimics the effects of ω-CmTx, a conotoxin which blocks Q-, N- and P-type channels (Wheeler *et al.*, 1994). However, until a useful preparation containing Q channels becomes available, the effect of RR on these VSCCs remains problematic.

The results of this study confirm and extend previous results concerning the effect of RR on Ca^{++} influx. More importantly, they delineate the inhibitory properties of this compound on N-, P- and NP-type VSCCs. These properties of RR suggest that it could prove to be an interesting and inexpensive tool with which to study Ca^{++} channels both *in vitro* and *in vivo*.

Acknowledgment

The authors gratefully acknowledge the research assistance of Robert Frew, Brian Hand and Lorne McDonald.

References

Allen, T. G. J., Sim, J. A. and Brown, D. A.: The whole-cell calcium current in acutely dissociated magnocellular cholinergic basal forebrain neurones of the rat. J. Physiol. (Lond.) **460:** 91–116, 1993.

Arias, C. and Tapia, R.: Differential calcium dependence of γ-aminobutyric acid and acetylcholine release in mouse brain synaptosomes. J. Neurochem. **47:** 396–404, 1986.

Ballesta, J. J., Palmero, M., Hidalgo, M. J., Gutierrez, L. M., Reig, J. A., Viniegra, S. and Garcia, A. G.: Separate binding and functional sites for ω-conotoxin and nitrendipine suggest two types of calcium channels in bovine chromaffin cells. J. Neurochem. **53:** 1050–1056, 1989.

Bertolino, M. and Llinás, R. R.: The central role of voltage-activated and receptor-operated calcium channels in neuronal cells. Annu. Rev. Pharmacol. Toxicol. **32:** 399–421, 1992.

Bowman, D., Alexander, S. and Lodge, D.: Pharmacological characterization of the calcium channels coupled to the plateau phase of KCl-induced intracellular free Ca^{2+} elevation in chicken and rat synaptosomes. Neuropharmacology **32:** 1195–1202, 1993.

De Feo, P. A., Mangano, T. J., Adams, M. E. and Keith, R. A.: Inhibition of ω-conotoxin GVIA insensitive neurotransmitter release by ω-AgaIVA. Pharmacol. Commun. **1:** 273–278, 1993.

De Luca, A., Li, C. G., Rand, M. J., Reid, J. J., Thaina, P. and Wong-Dusting, H. K.: Effects of ω-conotoxin GVIA on autonomic neuroeffector transmission in various tissues. Br. J. Pharmacol. **101:** 437–447, 1990.

Dooley, D. J., Lupp, A., Herting, G. and Osswald, H.: ω-Conotoxin GVIA and pharmacological modulation of hippocampal noradrenaline release. Eur. J. Pharmacol. **148:** 261–266, 1988.

Franco-Cereceda A., Lou Y.-P. and Lundberg J. M.: Ruthenium red differentiates between capsaicin and nicotine effects on cardiac sensory nerves. Acta Physiol. Scand. **137:** 457–458, 1989.

Geer, J. A., Dooley, D. J. and Adams, M. E.: K^+-stimulated $^{45}Ca^{2+}$ flux into rat neocortical mini-slices is blocked by ω-AgaIVA and the dual Na^+/Ca^{2+} channel blockers lidoflazine and flunarazine. Neurosci. Lett. **158:** 97–100, 1993.

Ginsberg, B. L. and Hirst, G. D. S.: The effects of adenosine on the release of the transmitter from the phrenic nerve of the rat. J. Physiol. (Lond.) **224:** 629–645, 1972.

Goddard, G. A. and Robinson, J. D.: Uptake and release of calcium by rat brain synaptosomes. Brain Res. **110:** 331–350, 1976.

Hansford, R. G.: Relation between cytostolic free Ca^{2+} concentration and the control of pyruvate dehydrogenase in isolated cardiac myocytes. Biochem. J. **241:** 145–151, 1987.

Herdon, H. and Nahorski, S. R.: Investigations of the roles of dihydropyridine and ω-conotoxin-sensitive calcium channels in mediating depolarisation-evoked endogenous dopamine release from striatal slices. Arch. Pharmacol. **340:** 36–40, 1989.

Hillyard, D. R., Monje, V. D., Mintz, I. M., Bean, B. P., Nadasdi, L., Ramachandran, J., Miljanich, G., Azimi-Zoonooz, A., McIntosh, J. M., Cruz, L. J., Imperial, J. S. and Olivera, B. M.: A new conus peptide ligand for mammalian presynaptic Ca^{2+} channels. Neuron **9:** 69–77, 1992.

Kamino, K., Ogawa, M., Uyesaka, N. and Inouye, A.: Calcium binding of synaptosomes isolated from rat brain cortex. IV. Effects of ruthenium red on the cooperative nature of calcium-binding. J. Membr. Biol. **26:** 345–356, 1976.

Keith, R. A., Mangano, T. J., Pacheco, M. A. and Salama, A. I.: Characterization of the effects of ω-conotoxin GVIA on the responses of voltage-sensitive calcium channels. J. Auton. Pharmacol. **9:** 243–252, 1989.

Knaus, H.-G., Streissnig, J., Koza, A. and Glossman, H.: Neurotoxic aminoglycoside antibiotics are potent inhibitors of [^{125}]ω-conotoxin GVIA binding to guinea-pig cerebral cortex. Naunyn-Schmiedeberg's Arch. Pharmacol. **336:** 583–586, 1987.

Llinás, R., Sugimori, M., Hillman, D. E. and Cherksey, B.: Distribution and functional significance of the P-type voltage dependent Ca^{2+} channel in the mammalian central nervous system. Trends Neurosci. **15:** 351–355, 1992.

Llinás, R., Sugimori, M., Lin, J. W. and Cherksey, B.: Blocking and isolation of a calcium channel from neurons in mammals and cephalopods utilizing a toxin fraction (FTX) from funnel-web spider poison. Proc. Natl. Acad. Sci. U.S.A. **86:** 1689–1693, 1989.

Lundy, P. M. and Frew, R.: Evidence of ω-conotoxin GVIA-sensitive Ca^{2+} channels in mammalian peripheral nerve terminals. Eur. J. Pharmacol. **156:** 325–330, 1988.

Lundy, P. M. and Frew, R.: Pharmacological characterization of voltage-sensitive Ca^{2+} channels in autonomic nerves. Eur. J. Pharmacol. **231:** 197–202, 1993.

Lundy, P. M. and Frew, R.: Effect of omega agatoxin IVA on autonomic neurotransmission. Eur. J. Pharmacol. **261:** 79–84, 1994.

Lundy, P. M., Frew, R., Fuller, T. W. and Hamilton, M. G.: Pharmacological evidence for an ω-conotoxin, dihydropyridine-insensitive neuronal Ca^{2+} channel. Eur. J. Pharmacol. **206:** 61–68, 1991.

Lundy, P. M., Hamilton, M. G. and Frew, R.: Pharmacological identification of a novel Ca^{2+} channel in chicken brain synaptosomes. Brain Res. **643:** 204–210, 1994.

Lundy, P. M., Stauderman, K., Goulet, J. C. and Frew, R.: Effect of ω-conotoxin GVIA on Ca^{2+} influx and endogenous acetylcholine release from chicken brain preparations. Neurochem. Int. **14:** 49- 54, 1989.

Maggi, C. A., Patacchini, R., Santicioli, P., Giuliani, S., Geppetti, P. and Meli, A.: Protective action of ruthenium red towards capsaicin desensitization of sensory fibres. Neurosci. Lett. **88:** 201–205, 1988.

Mangano, T. J., Patel, J., Salama, A. I. and Keith, R. A.: Inhibition of K^+ evoked [^{3}H]D-Aspartate release and neuronal calcium influx by verapamil, diltiazem and dextromethorphan: Evidence for non-L/non-N voltage-sensitive calcium channels. Eur. J. Pharmacol. **192:** 9–17, 1991.

Massieu, L. and Tapia, R.: Relationship of dihydropyridine binding sites with calcium dependent neurotransmitter release in synaptosomes. J. Neurochem. **51:** 1184–1189, 1988.

Miller, R. J.: Calcium channels in neurons. *In* Receptor Biochemistry and Methodology, ed. by J. C. Ventner and L. C. Harrison, pp. 161–246, Alan R. Liss Inc., New York, 1987.

Mintz, I. M., Adams, M. E. and Bean, B. P.: P-Type calcium channels in rat central and peripheral neurons. Neuron **9:** 85–95, 1992.

Meza-Ruiz, and Tapia, R.: [^{3}H]-GABA release in synaptosomal fractions after intracranial administration of ruthenium red. Brain Res. **154:** 163–166, 1978.

Nachshen, D. A. and Blaustein, M. P.: Some properties of potassium stimulated calcium influx in presynaptic nerve endings. J. Gen. Physiol. **76:** 709–728, 1980.

Pfrieger, F. W., Veselovsky, N. S., Gottman, K. and Lux, H. D.: Pharmacological characterization of calcium currents and synaptic transmission between thalamic neurons *in vitro*. J. Neurosci. **12:** 4347–4357, 1992.

Plummer, M. R., Logothetis, D. E. and Hess, P.: Elementary properties and pharmacological sensitivities of calcium channels in mammalian peripheral neurons. Neuron **2:** 1453–1463, 1989.

Pullar, I. A. and Findlay, J. D.: Effect of voltage-sensitive calcium channel antagonists on the release of 5-hydroxytryptamine from rat hippocampus *in vivo*. J. Neurochem. **59:** 553–559, 1992.

Rampe, D., Janis, R. A. and Triggle, D. A.: Bay K 8644, a 1,4-dihydropyridine Ca^{2+} channel activator: Dissociation of binding and functional effects in brain synaptosomes. J. Neurochem. **43:** 1688–1694, 1984.

Regan, L. J., Sah, D. W. Y. and Bean, B. P.: Ca^{2+} channels in rat central and peripheral neurons: High-threshold current resistant to dihydropyridine blockers and ω-conotoxin. Neuron **6:** 269–280, 1991.

Reynolds, I. J., Wagner, S. J. A., Snyder, S. H., Thayer, S. A., Olivera, B. M. and Miller, R. J.: Brain voltage-sensitive calcium channel subtypes differentiated by ω-conotoxin GVIA. Proc. Natl. Acad. Sci. U.S.A. **83:** 8804–8808, 1986.

Saccomano, N. A. and Ganong, A. H.: Diversity of neuronal calcium channels. *In* Annual Reports in Medicinal Chemistry, ed. by J. A. Bristol, pp. 33–42, Academic Press, San Diego, 1991.

Sitges, M.: Effect of organic and inorganic calcium channel blockers on γ-amino-*n*-butyric acid release induced by monensin and veratrine in the absence of external calcium. J. Neurochem. **53:** 436–441, 1989.

Skattebøl, A. and Triggle, D. J.: $^{45}Ca^{2+}$ uptake in rat brain neurons: Absence of sensitivity to the Ca^{2+} channel ligands nitrendipine and Bay K 8644. Can. J. Physiol. Pharmacol. **65:** 344–347, 1987a.

Skattebøl, A. and Triggle, D. J.: Regional distribution of calcium channel ligand (1,4-dihydropyridine) binding sites and $^{45}Ca^{2+}$ uptake processes in rat brain. Biochem. Pharmacol. **36:** 4163–4166, 1987b.

Suszkiw, J. B., Murawsky, M. M. and Fortner, R. C.: Heterogeneity of presynaptic calcium channels revealed by species differences in the sensitivity of synaptosomal ^{45}Ca entry to ω-conotoxin. Biochem. Biophys. Res. Commun. **145:** 1283–1286, 1987.

Suszkiw, J. B., Murawsky, M. M. and Shi, M.: Further characterization of phasic calcium influx in rat cerebrocortical synaptosomes: Inferences re-

garding calcium channel type(s) in nerve endings. J. Neurochem. **52:** 1260–1269, 1989.

TAKAHASHI, T. AND MOMLYAMA, A.: Different types of calcium channels mediate central synaptic transmission. Nature (Lond.) **366:** 156–158, 1993.

TAPIA, R.: Effects of drugs on neurotransmitter release: Experiments *in vivo* and *in vitro*. Neurosci. Biobehav. Rev. **9:** 391–397, 1985.

TAPIA, R. AND ARIAS, C.: Calcium transport and the release of neurotransmitters: Effects of drugs *in vivo* and *in vitro*. *In* Regulatory Mechanisms of Synaptic Transmission, ed. by R. Tapia and C. W. Cotman, pp. 169–186, Plenum Press, New York, 1981.

TAPIA, R., ARIAS, C. AND MORALES, E.: Binding of lanthanum ions and ruthenium red to synaptosomes and its effects on neurotransmitter release. J. Neurochem. **45:** 1464–1470, 1985.

TAPIA, R. AND MEZA-RUÍZ, G.: Inhibition by ruthenium red of the calcium-dependent release of [^{3}H]GABA in synaptosomal fractions. Brain Res. **126:** 160–166, 1977.

TURNER, T. J., ADAMS, M. E. AND DUNLAP, K.: Multiple Ca^{2+} channel types coexist to regulate synaptosomal neurotransmitter release. Proc. Natl. Acad. Sci. U.S.A. **90:** 9518–9522, 1993.

UCHITEL, O. D., PROTTI, D. A., SANCHEZ, V., CHERKSEY, B. D., SUGIMORI, M. AND LLINÁS, R. A.: P type voltage dependent calcium channel mediates presynaptic calcium influx and transmitter release in mammalian synapses. Proc. Natl. Acad. Sci. U.S.A. **89:** 3330–3333, 1992.

UGALDE-GARCIA, G. AND TAPIA, R.: Convulsions and wet-dog shakes produced by systemic or intrahippocampal administration of ruthenium red in the rat. Exp. Brain Res. **86:** 633–640, 1991.

WESSLER, I., DOOLEY, D. J., WERHAND, J. AND SCHLEMMER, F.: Differential effects of calcium channel antagonists (ω-conotoxin GVIA, nifedipine, verapamil) on the electrically-evoked release of [^{3}H]acetylcholine from the myenteric plexus, phrenic nerve and neocortex of rats. Naunyn-Schmiedeberg's Arch. Pharmacol. **341:** 288–294, 1990.

WHEELER, D. B., RANDALL, A. AND TSIEN, R. W.: Roles of N-type and Q-type Ca^{2+} channels in supporting hippocampal synaptic transmission. Science (Wash. DC) **264:** 107–111, 1994.

WOOD, N., WINTER, J., JAMES, I. F., RANG, H. P., YEATES, J. AND BEVAN, S.: Capsaicin-induced ion fluxes in dorsal root ganglion cells in culture. J. Neurosci. **8:** 3208–3220, 1988.

WOODWARD, J. J., REZAZADEH, S. M. AND LESLIE, S. W.: Differential sensitivity of synaptosomal calcium entry and endogenous dopamine release to ω-conotoxin. Brain Res. **475:** 141–145, 1988.

Send reprint requests to: Dr. Murray G. Hamilton, Département de Pharmacologie, Unité de Neurotoxicologie, Centre de Recherches du Service de Santé des Armées, 24, av. des Maquis du Grésivaudan, B.P. 87, 38702 La Tronche Cedex, France.