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RUTHENIUM RED INHIBITS SELECTIVELY CHROMAFFIN CELL CALCIUM CHANNELS

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Abstract—The effect of Ruthenium red (RR) on ionic currents and catecholamine secretion was studied in chromaffin cells. This polycation inhibited 59 mM potassium-stimulated $^{45}Ca^{2+}$ uptake in a concentration-dependent manner ($IC_{50} = 5 \pm 0.2\ \mu M$). This effect was more evident at extracellular calcium concentrations over 1 mM and was not abolished by neuraminidase pretreatment. RR also inhibited potassium-stimulated catecholamine secretion ($IC_{50} = 6 \pm 0.9\ \mu M$). These results were corroborated by patch-clamp in whole-cell recordings. RR inhibited chromaffin cell calcium currents ($IC_{50} = 7\ \mu M$) without affecting significantly either sodium or potassium currents. Radioligand binding studies in adrenomedullary plasma membranes showed that RR inhibited [^{125}I]ω-conotoxin GVIA binding but it had no effect on specific binding of [^{3}H]nitrendipine. The effect of the RR on calcium currents was additive with the inhibitory effect observed with 10 μM nitrendipine. The residual dihydropyridine-resistant calcium current was inhibited with a potency similar to that determined under control conditions in the absence of nitrendipine. These results demonstrate that RR selectively inhibits calcium channels; however, this polycation was not selective for a particular calcium channel subtype.

Key words: chromaffin cell, calcium channel, ruthenium red, polycation, neurosecretion, ionic currents

The hexavalent polycationic dye ruthenium red, $[(NH_3)_5Ru\text{-}O\text{-}Ru(NH_3)_4\text{-}O\text{-}Ru(NH_3)_5]Cl_6 \quad 4H_2O$ (RR§), has been used as a histochemical stain for acidic glycosaminoglycans [1]. Recently, the interaction of RR with calcium binding proteins has been reported [2]. RR has also been found to inhibit Ca^{2+}-induced Ca^{2+} release from skeletal or cardiac muscle [3], Ca^{2+}-dependent ATPases [4] and mitochondrial calcium accumulation [5].

In neural systems micromolar concentrations of this polycation inhibit calcium uptake and neurotransmitter secretion in nerve endings [6]. The inhibitory effect of RR on La^{3+} binding in synaptosomes suggested that RR could be acting on the voltage-dependent calcium channel [7]. Recently, it has been shown that RR blocks voltage-gated Ca^{2+} currents in neurons isolated from mouse sensory ganglia [8]. In addition, RR also inhibits calcium uptake and neuropeptide release evoked by different agonists in dorsal root ganglion cells in culture [9, 10].

The existence of at least two types of calcium channel, N- and L-subtypes, was initially demonstrated in chromaffin cells according to their functional and pharmacological properties [11]. Recently, the additional presence of a P-type calcium channel has been reported in chromaffin cells according to its sensitivity to the spider toxin FTX [12, 13]. The possible participation of these calcium channels in the catecholamine secretory process has been extensively studied in the last decade by using different organic and inorganic calcium antagonists [14, 15].

Organic calcium antagonists inhibit partially, and to a variable degree, both calcium uptake and catecholamine secretion in bovine adrenomedullary chromaffin cells, suggesting the presence of different calcium channel subtypes. For instance, the dihydropyridine antagonist nitrendipine that blocks L-type calcium channels inhibits approximately 60% of the potassium-stimulated $^{45}Ca^{2+}$ uptake [16]; however, differences in sensitivity to dihydropyridine of chromaffin cell calcium channels have been demonstrated in respect to species [17] or to the membrane potential [18].

The ω-conotoxin GVIA (ω-CgTx) that inhibits more specifically N-type calcium channels also blocks partially calcium uptake in chromaffin cells [16]. However, the inorganic cations inhibit completely the evoked catecholamine release and calcium uptake in both perfused adrenal glands [19] and cultured chromaffin cells [16], Zn^{2+} and Cd^{2+} being the most potent [18]. Probably these inorganic cations block the Ca^{2+} binding site(s) at the different calcium channel subtypes. In contrast, other divalent cations such as Sr^{2+} and Ba^{2+} can permeate calcium channels easily suggesting a high discriminatory capacity of the ionic pore of the channel. In addition to this activity on calcium channel activity, permeable and blocking divalent cations can modulate the binding of both nitrendipine and ω-CgTx [20, 21] suggesting a site for these organic ligands closely

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§ Abbreviations: ω-CgTx, ω-conotoxin GVIA; DMEM, Dulbecco's modified Eagle's medium; FTX, funnel-web spider toxin; RR, Ruthenium red; K/H, Krebs/HEPES.

related to the calcium binding site(s) at the different calcium channel subtypes.

The relative role of the different types of calcium channel in the secretory process in the chromaffin cell and other excitatory systems has not yet been determined. A polycationic molecule such as RR with a more complex structure than the divalent cations habitually used as calcium channel blockers is an interesting tool for exploring calcium channel function in chromaffin cells and other excitable cells.

In the present work, we were prompted to study the effect of RR on chromaffin cell ionic channels and we focussed on its inhibitory effect on calcium channels. We report that RR inhibits both dihydropyridine-sensitive and -insensitive chromaffin cell calcium channels with a similar potency, however it has a different effect on the binding of calcium channel ligands.

MATERIALS AND METHODS

Materials. The radioisotopes $^{45}Ca^{2+}$ (sp. act. 10–40 mCi/mmol) and [^{125}I]ω-conotoxin were obtained from Amersham International (U.K.); [^{3}H]noradrenaline (14 Ci/mmol) and [^{3}H]nitrendipine (78.3 Ci/mmol) were from NEN, Du Pont (Boston, MA, U.S.A.). Nitrendipine was a gift from Prof. F. Hoffmeister (Bayer AG; Wuppertal, Germany). RR, ω-CgTx, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum and neuraminidase (EC 3.2.1.18) type VI were from the Sigma Chemical Co. (Poole, U.K.). Collagenase (EC 3.4.24.3) was from Boehringer Mannheim (Germany). Percoll was from Pharmacia (Uppsala, Sweden). Other chemicals were of analytical grade from Sigma or Merck (Darmstadt, Germany).

Cell isolation, culture and neuraminidase treatment. Chromaffin cells were prepared from bovine adrenal glands by collagenase digestion [22] and purified from debris and erythrocytes using a percoll gradient. Cells were maintained in monolayer cultures at a density of 250,000 cells/cm^2 using DMEM supplemented with 10% fetal calf serum, 10 μM cytosine arabinoside to prevent fibroblast proliferation, and penicillin (25,000 IU/L) and streptomycin (25 mg/L). Cell were maintained at 37° in a humidified incubator under an atmosphere of 95% air and 5% CO_2. The neuraminidase treatment of chromaffin cells was carried out with 0.15 U/mL of neuraminidase type VI from *Clostridium perfringens* for 3 hr in DMEM.

[^{3}H]*Noradrenaline release.* Chromaffin cells were loaded with [^{3}H]noradrenaline (1 μCi/mL) in DMEM for 3–4 hr in the presence of 1 mM ascorbic acid. Then, culture medium was replaced by Krebs/HEPES (K/H) solution with the following composition (in mM): NaCl 134, KCl 4.7, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5, glucose 11, ascorbic acid 0.56 and HEPES 15. The solution was equilibrated with pure oxygen and the pH adjusted to 7.4. Cells were washed six times with this medium previous to incubation of cultures with different concentrations of RR for 5 min in K/H basal. Then, catecholamine secretion was induced by cell depolarization with high potassium (K^+ 59 mM, replacing isosmotically the concentration of NaCl by KCl) for 5 min. After that, media were collected and cells were lysed with 2% SDS. Secreted and total catecholamines were quantified by liquid scintillation spectrometry.

$^{45}Ca^{2+}$ *uptake.* Cells cultured in 24-well plates were washed three times with K/H solution. $^{45}Ca^{2+}$ uptake was determined by incubating chromaffin cells for 30 sec, or the indicated times, in the presence of $^{45}Ca^{2+}$ (4 μCi/mL) in basal or high potassium K/H solution. In the experiments with RR, cells were previously incubated with the polycation for 5 min in K/H solution. After the experiments, cells were washed once with a K/H solution lacking $CaCl_2$ and KH_2PO_4 and containing 2 mM $LaCl_3$ and 2 mM EGTA. Then, cells were quickly washed five times with a cold K/H solution. Cells were lysed with 2% SDS and the radioactivity incorporated into the cells was quantified by liquid scintillation spectrometry.

Binding experiments. Adrenomedullary plasma membranes were purified through a sucrose gradient as described previously [16]. For ω-CgTx binding experiments, membranes (10 μg/mL) were incubated with increasing concentrations of RR in the presence of 10 pM [^{125}I]ω-conotoxin. For nitrendipine binding, membranes were washed with EDTA and EGTA as described previously [21], and then membranes (50 μg/mL) were incubated with increasing concentrations of RR in the presence of 0.8 nM [^{3}H]-nitrendipine. In both cases, incubations were carried out for 1 hr at 25°. At the end of the incubation period, samples were filtered under vacuum through Whatmann GF/C glass fiber filters, washed and counted as reported previously [23]. Non-specific binding was determined in the presence of 50 nM unlabeled ω-CgTx or 10 μM unlabeled nitrendipine. Data were fitted to a sigmoid curve using a non-linear least square program.

Recording and analysis of the ionic currents. Inward currents were recorded using the "whole-cell" configuration of the patch-clamp technique with an Axopatch-1D amplifier (Axon Instruments) using the standard technique [24]. External solution contained (in mM): 135 NaCl, 5 KCl, 5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES-NaOH, pH 7.2 supplemented with 0.39 g/L glucose. Soft glass patch-clamp electrodes were filled with (in mM): 100 CsCl, 3 MgATP, 15 EGTA, 10 NaCl, 20 tetraethylammonium-Cl, 20 HEPES-CsOH pH 7.2. For measuring potassium outward currents, cesium and tetraethylammonium in internal solution were substituted by potassium. Electrode resistances ranged from 4 to 10 MΩ when filled with internal solution. Series resistances were compensated by 80%. RR was delivered near the cell by a puffer pipette. Recordings were done at room temperature (22°). Currents were digitized at 8 kHz by using pCLAMP software with a Labmaster interface (Axon Instruments), and stored on the hard disk of a PC/AT computer for subsequent analysis. Linear leak and capacitive currents were eliminated by a P/4 protocol. Data manipulation and analysis were done by using programs written in QBASIC language (Microsoft).

RESULTS

Effect of RR on $^{45}Ca^{2+}$ uptake and [^{3}H]noradrenaline secretion

In chromaffin cells, voltage-dependent calcium

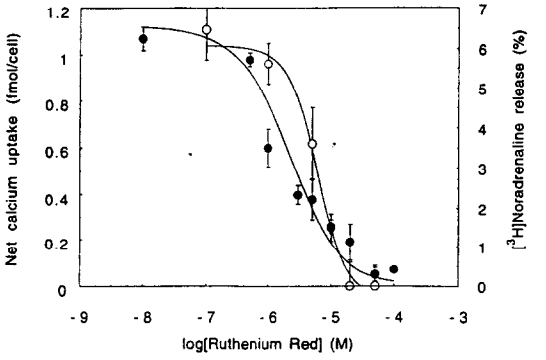

Fig. 1. Concentration-dependent inhibition by RR of $^{45}Ca^{2+}$ uptake (●) and [^{3}H]noradrenaline secretion (○) in cultured chromaffin cells. Chromaffin cells (5×10^5/assay) were incubated for 30 sec in basal or high potassium (59 mM) K/H solution without or in the presence of increasing concentrations of RR. Reaction was stopped with a La^{3+}-EGTA K/H solution and cells were quickly washed with K/H solution and lysed with 2% SDS. In order to quantify catecholamine secretion, cells (5×10^5/assay) were incubated with [^{3}H]noradrenaline and then treated with basal or high (59 mM) potassium K/H solution without or in the presence of increasing concentrations of RR for 5 min, media were collected to quantify secreted catcholamines and cells were lysed with 2% SDS in order to evaluate the [^{3}H]noradrenaline content. Secretion is expressed as a percentage of the total content. Data are given as the means ± SEM of three experiments.

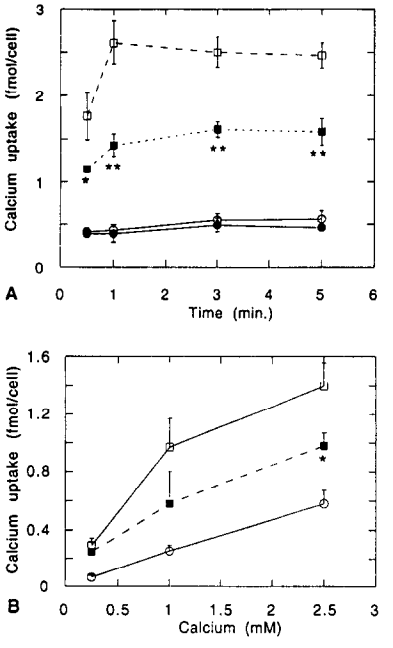

Fig. 2. Inhibition of high potassium-induced $^{45}Ca^{2+}$ uptake by RR. (A) Time-dependent effect; (B) calcium concentration-dependent effect. Chromaffin cells (5×10^5/assay) were incubated in the presence of $^{45}Ca^{2+}$ (4 μCi/mL) for the indicated times (A) in basal (○, ●) or high potassium (59 mM) (□, ■) K/H solution at 2.5 mM extracellular calcium, without (○, □) or in the presence of 5 μM RR (●, ■). (B) Chromaffin cells were incubated in the presence of $^{45}Ca^{2+}$ for 30 sec at the indicated extracellular calcium concentration, in basal (○) or high potassium (59 mM) (□, ■) K/H solution, without (○, □) or in the presence of 5 μM RR (■). Reactions were stopped with a La^{3+}-EGTA K/H solution. Cells were lysed with 2% SDS. Data are given as the means ± SEM of three experiments. Stimulated calcium uptake values in the presence of RR which differ significantly (Student's *t*-test) from those obtained under control conditions are indicated by asterisks: *$P < 0.02$, **$P < 0.002$.

channels are activated by nicotinic agonists or directly by membrane depolarization using a high potassium solution (59 mM). Initially, in order to study the possible effect of RR on calcium channels, cells were depolarized with 59 mM K^+ for 30 sec and $^{45}Ca^{2+}$ uptake was evaluated in the presence of different concentrations of RR. As is shown in Fig. 1, RR concentration-dependently inhibited (IC_{50} = 5.0 ± 0.2 μM) of stimulated $^{45}Ca^{2+}$ uptake. This inhibitory effect was also seen with the nicotinic agonist 1,1-dimethyl-4-phenyl-piperazinium. Net $^{45}Ca^{2+}$ uptake induced by 10 μM 1,1-dimethyl-4-phenyl-piperazinium was 0.46 fmol/cell, slightly lower than that observed with 59 mM K^+; this uptake was further reduced to 0.12 fmol/cell in the presence of 1 μM RR.

In chromaffin cells, the intracellular rise in calcium concentration triggers the release of catecholamines. Accordingly, it was reasonable to expect a parallel inhibitory effect of RR on the secretory process. It was found (Fig. 1) that RR produced a concentration-dependent decrease in the [^{3}H]noradrenaline secretion evoked by depolarization with 59 mM K^+. The calculated IC_{50} was 6.0 ± 0.9 μM, close to that observed in the former experiments on $^{45}Ca^{2+}$ uptake.

The time course of the high potassium-induced $^{45}Ca^{2+}$ uptake (Fig. 2A) showed that RR at a concentration of 5 μM had an inhibitory effect over the whole range studied, decreasing the uptake in the stationary phase by 50%. Neither basal calcium uptake nor basal catecholamine secretion were modified in the presence of RR.

If RR was acting just as a competitive inhibitor, an increase in the extracellular calcium concentration should overcome the effect of the polycation. However, as illustrated in Fig. 2B the inhibitory effect of RR was higher at an extracellular calcium concentration of over 1 mM, and the inhibition being almost the same at 2.5 mM calcium.

The possible interaction of RR with sialic acid residues in the plasmalemmal membrane has been suggested to explain some of the effects of RR [25]. In order to test this possibility, chromaffin cells were incubated with neuraminidase under conditions where RR actions, presumably mediated through sialic acid interactions, are abolished [26]. In these experiments, net 59 mM K^+-stimulated $^{45}Ca^{2+}$ uptake was partially inhibited from 0.76 fmol/cell, under control conditions, to 0.60 fmol/cell after neuraminidase treatment. However, the inhibitory effect of RR on the remaining net calcium uptake was not significantly modified by neuraminidase,

showing an additive effect. In the presence of 10 μM RR, net calcium uptake was 0.37 and 0.28 fmol/cell under control conditions and upon neuraminidase treatment, respectively. This result suggests that the effect of RR on both calcium uptake and catecholamine release is not the consequence of an interaction with sialic acid-containing components.

In order to study the reversibility of the effect of RR, after a 5-min pretreatment, net calcium uptake was measured following wash out of the polycation. Under control conditions, 10 μM RR inhibited net $^{45}Ca^{2+}$ uptake by 70%, from 1.02 to 0.34 fmol/cell. Upon a 30-min wash out, during which the K/H solution was replaced three times, net $^{45}Ca^{2+}$ uptake was recovered to 0.71 fmol/cell which corresponded to 30% inhibition. The partial reversibility of the effect of RR indicates that it is produced by reversible binding.

Selective effect of RR on chromaffin cell calcium channels

In order to confirm the effect of RR on calcium channels and to explore the specificity of action of such an effect, we measured inward currents by using the patch-clamp technique in the "whole-cell" configuration. Depolarizing pulses of 42 msec were applied from a holding potential of −80 mV. Under these conditions, the transient inward current just after the depolarizing step (Fig. 3A) corresponds mostly to the fast sodium current that is abolished in the presence of 1 μM tetrodotoxin. Since sodium channels inactivate quickly, the late sustained component corresponds to current carried through voltage-activated calcium channels. The later currents probably represent the activity of both N- and L-type calcium channels described in chromaffin cells [11]. The FTX-sensitive calcium channel reported in chromaffin cells [12, 13] will be referred to hereafter as part of the DHP-insensitive calcium currents. As shown in Fig. 3A, B, 1 μM RR decreased calcium currents without significant effects on sodium currents. The determined IC_{50} for the effect of RR was 7 μM, which is in fair agreement with that observed in $^{45}Ca^{2+}$ uptake experiments. Calcium currents were totally recovered after a 2 min wash out of the polycation. Since peak current is contaminated by activating calcium current, it is worth noticing that the magnitude of the effects on peak currents should be considered only as an upper limit of the inhibition of sodium channels. On the other hand, RR had no significant effect on voltage-dependent potassium currents (data not shown).

The possible preference of RR for inhibiting a specific type of calcium channel (N-, P- or L-type) was addressed by measuring the inhibitory effect of RR on calcium currents in the presence of 10 μM nitrendipine that suppresses currents through L-type calcium channels [27]. As shown in Fig. 2C, either 1 μM RR or 10 μM nitrendipine inhibited calcium currents by 25%. However, the inhibitory effect of RR on the residual current in the presence of the dihydropyridine is of the same magnitude (26%) as under control conditions. This result suggests that RR inhibits both dihydropyridine-sensitive and -insensitive calcium channels apparently with a very similar potency. Moreover, RR reduced Ca^{2+}

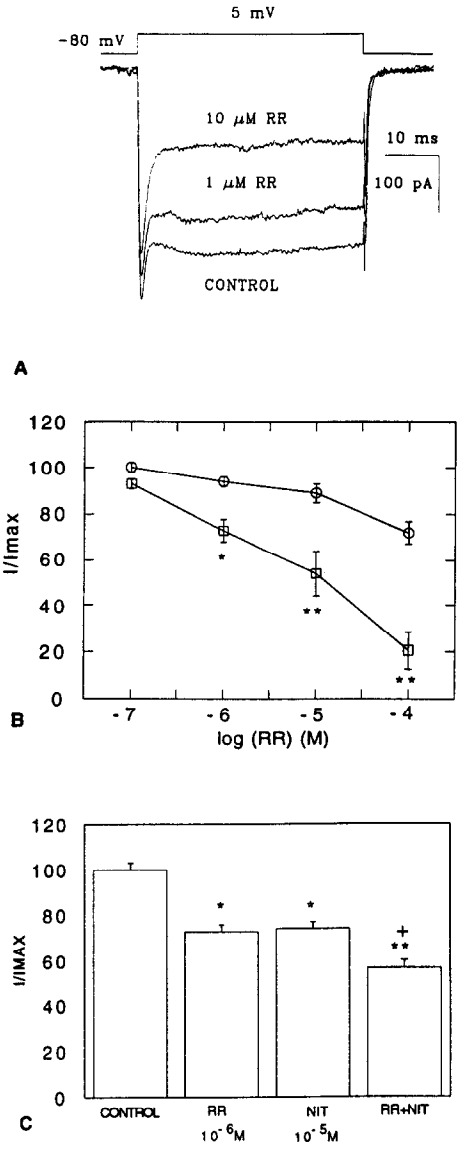

Fig. 3. Effect of RR on calcium and sodium currents in cultured chromaffin cells. Whole cell inward currents from a bovine chromaffin cell when 40 msec depolarizing pulses were applied from −80 mV to +5 mV. (A) Superimposed current traces in the absence (control) or the presence of RR (1 and 10 μM). Linear capacitive and leak currents were substracted. (B) Dose–response relationship for the effect of different concentrations of RR on the early peak (Na^+ current) (○) or the plateau phase (Ca^{2+} current) (□). Data are means ± SEM of experiments performed in six cells from different batches. (C) Additive effect of 1 μM RR and 10 μM nitrendipine (NIT) on the inhibition of Ca^{2+} current. Whole cell Ca^{2+} currents were recorded from cells exposed to either 10 μM nitrendipine or 1 μM RR or in the presence of both drugs at the indicated concentrations. Data are means ± SEM of experiments performed with seven cells from different preparations. In (B) data that differ significantly (Student's *t*-test) from the corresponding sodium current are indicated by asterisks: *$P < 0.002$, **$P < 0.001$. In (C) data that differ significantly from control are indicated by asterisks: *$P < 0.002$, **$P < 0.001$. Data obtained in the presence of both nitrendipine and RR differ significantly (+$P < 0.002$) from those obtained with either alone.

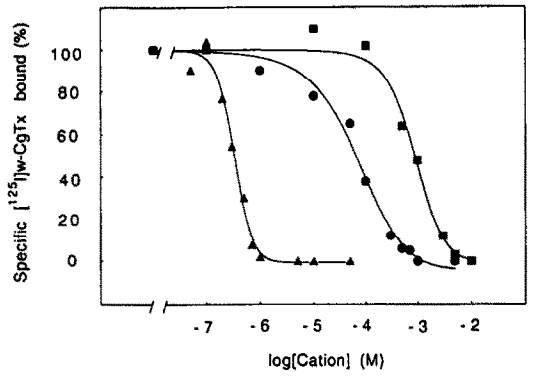

Fig. 4. Concentration-dependent inhibition of [^{125}I]ω-conotoxin binding to adrenomedullary plasma membranes by RR, Ca^{2+} and Cd^{2+}. Plasma membranes (100 μg protein/mL) were incubated with 10 pM [^{125}I]ω-conotoxin in the absence and in the presence of increasing concentrations of RR (▲), Ca^{2+} (■) or Cd^{2+} (●) for 1 hr at 25°. Values are expressed as percentage of specific binding in absence of cations. Data are means of two experiments performed in duplicate. Experimental conditions were as described in Materials and Methods. Data were fitted to a sigmoid curve using a non-linear least square program.

currents to the same extent if the membrane potential was held at −40 mV when a minor contribution of N-type calcium channels should be expected (data not shown).

Effect of RR on the specific [^{3}H]*nitrendipine and* [^{125}I]ω*-conotoxin binding to adrenomedullary plasma membranes*

In brain membranes and other tissues, cations that act as calcium agonists or antagonists can modulate both [^{125}I]ω-conotoxin [20] and [^{3}H]nitrendipine binding [21]. The effect of RR on [^{125}I]ω-conotoxin binding was studied and we observed that RR decreased toxin binding in a concentration-dependent manner (IC_{50} = 0.35 μM) (Fig. 4). In this experiment, the effect of Ca^{2+} and Cd^{2+} in modulating the binding is also shown. The potency of the effect of the divalent cations is substantially lower (IC_{50} = 850 and 80 μM, respectively) than that observed with RR. In contrast, RR in the range 0.1–50 μM was unable to displace the specific [^{3}H]nitrendipine binding to adrenomedullary plasma membranes. The calcium requirement of the dihydropyridine binding in this tissue has been previously reported by using EGTA washed membranes [23]. At this point we decided to investigate whether RR, as had been reported for divalent cations, could modulate [^{3}H]-nitrendipine binding in membranes treated with EDTA and EGTA [21]. The result of these experiments showed that RR, between 1 and 10 μM, had no effect on specific [^{3}H]nitrendipine binding to adrenomedullary plasma membranes under conditions where Ca^{2+} at 500 μM duplicated the specific binding (Table 1). This result suggests that RR has properties different to those of divalent cations in respect to modulation of [^{3}H]nitrendipine binding to the dihydropyridine receptor associated with the L-type calcium channel.

Table 1. Effect of RR and Ca^{2+} on specific [^{3}H]nitrendipine binding to adrenomedullary plasma membranes

	cpm	%
Control	202 ± 45	100
RR (1 μM)	232 ± 14	114
RR (5 μM)	208 ± 28	102
RR (10 μM)	189 ± 37	94
Ca^{2+} (500 μM)	440 ± 35*	218

Data are means ± SEM of three experiments using 0.8 nM [^{3}H]nitrendipine and a concentration of membrane protein of 0.05 mg/0.1 mL (final volume). Membranes were washed in the presence of EDTA and EGTA as described previously [21].

Experimental conditions were as described in Materials and Methods.

* Statistically significant from control (Student's *t*-test) ($P < 0.01$).

These experiments demonstrate that RR has the same behavior as some divalent cations which inhibit ω-conotoxin binding, but seems to act differently with respect to the binding of dihydropyridines.

DISCUSSION

The present work demonstrates that micromolar concentrations of the polycationic dye RR inhibit calcium currents in chromaffin cells. Different cations such as Cd^{2+} and La^{3+} also block calcium currents; however, these can modulate dihydropyridine binding [21] while RR has no effect, suggesting some differences in their interaction with the channel.

The possible relationship between the observed inhibitory effect of RR and interaction with sialic acid residues on the channel or closely related components can be ruled out as neuraminidase treatment did not reverse the effect. However, the effect of RR was reversible after washing out the polycation, both in the $^{45}Ca^{2+}$ uptake experiments and in the electrophysiological recordings in which it was shown that calcium currents were fully recovered by washing after RR application.

Given the polycationic nature of RR, it could be argued that it exhibits its effects by screening negative surface charges.The apparent inhibition could have been due to a shift in the activation curve of calcium channels. However, this is not the case since no shift in the *I/V* relationships was seen in the presence of RR; maximal calcium current was found to be always around +10 mV (data not shown).

An effect of RR on Na^+–Ca^{2+} exchange, increasing intracellular calcium concentration and therefore inhibiting calcium current, could not be totally ruled out. Nevertheless, some facts argue against such a possibility: first, Na^+–Ca^{2+} exchange is probably electrogenic and the effect of RR is insensitive to both holding and test potential; and second, the interaction of RR with ω-conotoxin binding sites strongly suggests that RR is acting on calcium channels.

Another interesting point is the fact that RR affects calcium currents equally regardless of the voltage applied (data not shown), indicating that the site of action of RR is out of the membrane electric field. In spite of the much more complex structure of RR when compared with other blocking or permeating cations, such a site might share one or more loci with that reported for the blocking effect of either Ca^{2+} in guinea pig ventricular cells calcium channels [28] or Cd^{2+} in cat sensory neurone calcium channels [29]. Due to the relatively small differences in radii among Ca^{2+} (0.99 Å) and other divalent cations (0.65–1.35 Å) [30], it is reasonable to suppose that they have a common binding site(s) in the calcium channel; however, the more complex structure of the RR molecule (15 Å in length and 8 Å in width) [31] makes it probable that the interaction of the polycation is a little different.

The inhibitory effect of RR on calcium currents and consequently on catecholamine secretion is selective. We report for the first time in the present work that sodium or potassium currents are not affected by the polycation in the same studied range of concentration. Nevertheless, no differences in the inhibitory potency between dihydropyridine-sensitive and -insensitive calcium channels were observed. However, the different effect of RR on the binding of [^{3}H]nitrendipine and [^{125}I]ω-conotoxin might indicate a different interaction of RR in each one of these calcium channels subtypes. Further experiments need to be carried out to characterize the nature of this interaction.

There is in fact great interest in the discovery or synthesis of calcium channel subtype-specific toxins and derivatives, in order to investigate the relative role of these channels in allowing intracellular calcium-mediated actions. The effect of some organic polycations such as the spider toxin FTX [32] and polyamines [33] on voltage-activated calcium channels has been reported recently. The results described here, with the inorganic polycation RR, suggest that this compound or closely related molecules could be of interest for exploring Ca^{2+} channel activity.

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REFERENCES

1. Gustafson GT and Pihl E, Staining of mast cells acid glycosaminoglycans in ultrathin sections by ruthenium red. *Nature* **216**: 697–698, 1967.
2. Charuk GHM, Pirraglia CA and Reithmeier RAF, Interaction of ruthenium red with Ca^{2+}-binding proteins. *Anal Biochem* **188**: 123–131, 1990.
3. Volpe P, Salviati G and Chu A, Calcium-gated calcium channels in sarcoplasmic reticulum of rabbit skinned skeletal muscle fibers. *J Gen Physiol* **87**: 289–303, 1986.
4. Watson EL, Vicenzi FF and Davis PW, Ca^{2+}-activated membrane ATPase: selective inhibition by ruthenium red. *Biochim Biophys Acta* **249**: 606–610, 1971.
5. Ash GR and Bygrave FL, Ruthenium red as a probe in assessing the potential of mitochondria to control intracellular calcium in liver. *FEBS Lett* **78**: 166–168, 1977.
6. Tapia R and Meza-Ruiz G, Inhibition by ruthenium red of the calcium-dependent release of [^{3}H]GABA in synaptosomal fractions. *Brain Res* **126**: 160–166, 1977.
7. Tapia R, Arias C and Morales E, Binding of lanthanum and ruthenium red to synaptosomes and its effects on neurotransmitter release. *J Neurochem* **45**: 1467–1470, 1985.
8. Duchen MR, Ca^{2+}-dependent changes in the mitochondrial energetics in single dissociated mouse sensory neurons. *Biochem J* **283**: 41–50, 1992.
9. Franco-Cereceda A, Rydh M and Dalsgaard C-J, Nicotine and capsaicin, but not potassium-evoked CGP-release from cultured guinea-pig spinal ganglia is inhibited by ruthenium red. *Neurosci Lett* **137**: 72–74, 1992.
10. Cahill CM, White TD and Sawyok J, Involvement of calcium channels in depolarization-evoked release of adenosine from spinal cord synaptosomes. *J Neurochem* **60**: 886–893, 1993.
11. Bossu J-L, De Waard M and Feltz A, Two types of calcium channels are expressed in adult bovine chromaffin cells. *J Physiol* **437**: 621–634, 1991.
12. Duarte CB, Rosario LM, Sena CM and Carvalho AP, A toxin fraction (FTX) from the funnel-web spider poison inhibits dihydropyridine-insensitive Ca^{+2} channels coupled to catecholamine release in bovine adrenal chromaffin cells. *J Neurochem* **60**: 909–913, 1993.
13. Gandía L, Albillos A and García AG, Chromaffin cells possess FTX-sensitive calcium channels. *Biochem Biophys Res Commun* **194**: 671–676, 1993.
14. Garcia AG, Sala F, Reig JA, Viniegra S, Frias J, Fonteriz R and Gandia L, Dihydropyridine BAY-K-8644 activates chromaffin cell calcium channels. *Nature* **309**: 69–71, 1984.
15. Artalejo CR, Lopez MG, Castillo CF, Moro MA and García AG, L-type calcium channels and adrenomedullary secretion. In: *The Calcium Channel: Structure, Function and Implications* (Eds. Morad M, Nayler W, Kazda S and Schramm M), pp. 347–362. Springer, Berlin, 1988.
16. Ballesta JJ, Palmero M, Hidalgo MJ, Gutierrez LM, Reig JA, Viniegra S and García AG, 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.
17. Gandía L, Michelena P, De Pascual R, Lopez MG and García AG, Different sensitivities to dihydropyridines of catecholamine release from cat and ox adrenals. *Neuroreport* **1**: 119–122, 1990.
18. Lopez MG, Moro MA, Castillo CF, Artalejo CR and García AG, Variable voltage-dependent blocking effects of nitrendipine, verapamil, diltiazem, cinnarizin and cadmium on adrenomedullary secretion. *Br J Pharmacol* **96**: 725–731, 1989.
19. Gandía L, Lopez MG, Fonteriz RI, Artalejo CR and García AG, Relative sensitivity of chromaffin cell calcium channels to organic and inorganic calcium antagonists. *Neurosci Lett* **77**: 333–338, 1987.
20. Abe T, Koyano K, Saisu H, Nishiuchi Y and Sakakibara S, Binding of ω-conotoxin to receptor sites associated with the voltage-sensitive calcium channel. *Neurosci Lett* **71**: 203–208, 1986.
21. Gould RJ, Murphy KMM and Snyder SH, [^{3}H]-Nitrendipine-labeled calcium channels discriminate inorganic calcium agonists and antagonists. *Proc Natl Acad Sci USA* **79**: 3656–3660, 1982.
22. Almazan G, Aunis D, Garcia G, Montiel C, Nicolas GP and Sanchez-Garcia P, Effects of collagenase on the release of [^{3}H]noradrenaline from bovine cultured adrenal chromaffin cells. *Br J Pharmacol* **81**: 599–610, 1984.

23. Ballesta JJ, García AG, Gutierrez LM, Hidalgo MJ, Palmero M, Reig JA and Viniegra S, Separate [^{3}H]-nitrendipine binding sites in mitochondria and plasma membranes of bovine adrenal medulla. *Br J Pharmacol* **101**: 21–26, 1990.
24. Hamill OP, Marty A, Neher E, Sakmann B and Sigworth FJ, Improved patch-clamp techniques for high resolution current from cells and cell-free membrane patches. *Pflugers Arch* **391**: 85–100, 1981.
25. Baux G, Simonneau M and Tauc L, Blocking action of ruthenium red on cholinergic and non-cholinergic synapses: possible involvement of sialic-acid containing substrates in neurotransmission. *Brain Res* **152**: 633–638, 1978.
26. Wieraszku A, Evidence that ruthenium red disturbs the synaptic transmission in the rat hippocampal slices through interacting with sialic acid residues. *Brain Res* **378**: 120–126, 1986.
27. Mintz IM, Adams ME and Beam BP, P-type calcium channels in rat central and peripheral neurons. *Neuron* **9**: 85–95, 1992.
28. Lansman JB, Hess P and Tsien RW, Blockade of current through single channels by Cd^{+2}, Mg^{+2} and Ca^{+2}. Voltage and concentration dependence of calcium entry into the pore. *J Gen Physiol* **407**: 443–452, 1986.
29. Taylor RW, Permeation of barium and cadmium through slowly inactivating calcium channels in cat sensory neurons. *J. Physiol* **407**: 433–452, 1988.
30. Hille B, *Ionic Channels of Excitable Membranes*, 2nd Edn. Sinauer, 1992.
31. Cotton SA and Hart FA, *The Heavy Transition Elements*. Macmillan Press, 1975.
32. Cherskey BD, Sugimori M and Llinas R, Properties of calcium channels isolated with spider toxin, FTX. *Ann NY Acad Sci* 80–89, 1991.
33. Herman MD, Reuveny E and Narahashi T, The effect of polyamines on voltage-activated calcium channels. *J Physiol* **462**: 645–660, 1993.