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Permeation by zinc of bovine chromaffin cell calcium channels: relevance to secretion

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Abstract Zn^{2+} increased the rate of spontaneous release of catecholamines from bovine adrenal glands. This effect was Ca^{2+} independent; in fact, in the absence of extracellular Ca^{2+}, the secretory effects of Zn^{2+} were enhanced. At low concentrations (3–10 μM), Zn^{2+} enhanced the secretory responses to 10-s pulses of 100 μM 1,1-dimethyl-4-phenylpiperazinium (DMPP, a nicotinic receptor agonist) or 100 mM K^+. In the presence of DMPP, secretion was increased 47% above controls and in high-K^+ solutions, secretion increased 54% above control. These low concentrations of Zn^{2+} did not facilitate the whole-cell Ca^{2+} (I_{Ca}) or Ba^{2+} (I_{Ba}) currents in patch-clamped chromaffin cells. Higher Zn^{2+} concentrations inhibited the currents (IC_{50} values, 346 μM for I_{Ca} and 91 μM for I_{Ba}) and blocked DMPP- and K^+-evoked secretion (IC_{50} values, 141 and 250 μM, respectively). Zn^{2+} permeated the Ca^{2+} channels of bovine chromaffin cells, although at a much slower rate than other divalent cations. Peak currents at 10 mM Ba^{2+}, Ca^{2+}, Sr^{2+} and Zn^{2+} were 991, 734, 330 and 7.4 pA, respectively. Zn^{2+} entry was also evidenced using the fluorescent Ca^{2+} probe fura-2. This was possible because Zn^{2+} causes an increase in fura-2 fluorescence at the isosbestic wavelength for Ca^{2+}, i.e. 360 nm. There was a slow resting entry of Zn^{2+} which was accelerated by stimulation with DMPP or high-K^+ solution. The entry of Zn^{2+} was concentration dependent, slightly antagonized by 1 mM Ca^{2+} and completely blocked by 5 mM Ni^{2+}. The entry of Ca^{2+} evoked by depolarization with high-K^+ solution was antagonized by Zn^{2+}. We conclude that inhibition by Zn^{2+} of evoked catecholamine secretion is associated with blockade of Ca^{2+} entry through Ca^{2+} channels recruited by DMPP or K^+. However, the facilitation of secretion observed at low Zn^{2+} concentrations, or in the absence of Ca^{2+}, may be exerted at an intracellular site on the secretory machinery. This is plausible because Zn^{2+} permeates the bovine chromaffin cell Ca^{2+} channels and in this way gains access to the cytosol. In addition, we have established conditions for measuring Zn^{2+} transients in fura-2-loaded cells with a very high sensitivity, taking advantage of the high-affinity binding of Zn^{2+} to fura-2 and the modification of its fluorescence spectrum.

Key words Zinc · Chromaffin cell
Catecholamine release · Calcium channels
Cytosolic calcium · Fura-2

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Introduction

Zn^{2+} is a divalent transition ion involved in several physiological processes. Its participation in cell division, membrane stabilization, protection against free radical cytotoxicity, as well as in the activity of numerous metalloenzymes has been broadly described [21]. Zn^{2+} is naturally attached to cell membranes, especially in various regions of the central nervous system, i.e. olfactory bulb, pineal gland and hippocampus [3, 24]. Various neurological diseases have been imputed to a Zn^{2+} deficit [9]. Zn^{2+} has been suggested to act as a modulator of synaptic activity through the blockade of Na^+, K^+-ATPase, the inhibition of K^+ and Ca^{2+} channels, the regulation of γ-amino butyric $acid_A$ ($GABA_A$) or *N*-methyl D-aspartate (NMDA) receptors [11], or the induction of $GABA_B$-mediated synaptic potentials in the hippocampus [29].

Recent studies suggest that Zn^{2+} may interfere with the secretion processes of different secretory glands [4,

12]. The present study shows that, in addition to the expected blockade of evoked adrenal catecholamine release [13, 23], Zn^{2+} exhibits two unexpected effects: the increase of the spontaneous catecholamine release and the facilitation of evoked secretion. In order to understand the mechanisms involved in these peculiar effects, we have also explored the actions of Zn^{2+} on whole-cell Ca^{2+} (I_{Ca}) and and Ba^{2+} (I_{Ba}) currents through Ca^{2+} channels, as well as its ability to permeate those channels; this is possible because of the ability of Zn^{2+} to modify the fluorescence spectrum of fura-2.

Materials and methods

Perfusion of adrenal glands

Bovine adrenal glands, obtained from a local slaughterhouse within 20–30 min after the death of the animals, were brought to the laboratory in cold Krebs-TRIS solution. Retrograde perfusion was then carried out at a rate of 15 ml·min^{-1}, at 37°C with Krebs-HEPES solution (pH 7.4, continuously bubbled with pure O_2 of the following composition (mM): NaCl 144; KCl 5.9; $MgCl_2$ 1.2; $CaCl_2$ 2.5; HEPES 10; glucose 11. The glands were initially equilibrated with normal Krebs-HEPES solution for 60 min.

Catecholamine release from perfused glands

The spontaneous catecholamine output was studied through the sequential collection of samples of the fluid (normal Krebs-HEPES) emanating from the gland in acidified chilled tubes (0.05 N perchloric acid, final concentration). Zn^{2+} was introduced into the superfusion fluid at increasing concentrations and samples were continuously collected.

The catecholamine release was triggered by perfusing 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), a nicotinic receptor agonist, or by direct depolarization with high K^+ concentrations. In the K^+-enriched solutions, Na^+ was equiosmotically reduced to maintain isotonicity. Usually, a given gland was repeatedly stimulated by applying 10-s pulses of a given secretagogue.

Total catecholamine present in aliquots of each acidified sample collected were fluorometrically assayed without further purification, following the method of Shellenberger and Gordon [22]; appropriate standards of pure noradrenaline and adrenaline were used, at the proportions (40/60%) known to be present in the bovine adrenal medulla.

Preparation and culture of chromaffin cells

Bovine adrenal chromaffin cells were isolated and prepared as described by Moro et al. [19]. Cells were suspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 50 IU·ml^{-1} penicillin and 50 µg·ml^{-1} streptomycin. For patch-clamp measurements of currents, cells were plated on circular glass coverslips at a density of 5×10^4 cells·ml^{-1}. For fura-2 measurements of cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$), cells were plated on poly-L-lysine-coated glass coverslips which were individually placed in wells of a Costar plate, at a density of 5×10^5 cells/coverslip. Cells were maintained in an incubator at 37°C in a water-saturated, 5% CO_2/95% air atmosphere. Viability of cells was greater than 90%, as estimated by trypan blue exclusion. Experiments were performed in cells which had been in culture for 1–4 days.

Measurements and analysis of Ca^{2+}, Sr^{2+}, Ba^{2+} and Zn^{2+} currents

Membrane currents were measured with the patch-clamp technique [15] in the whole-cell configuration, using a List EPC-7 patch-clamp amplifier and pipettes of borosilicate glass with a resistance of 2–5 MΩ when filled with the standard Cs/tetraethylammonium (TEA) intracellular solution.

The external bath solution contained (in mM): 10 $BaCl_2$ (or 10 $CaCl_2$ or 10 $SrCl_2$ or 10 $ZnCl_2$), 1 $MgCl_2$, 137 NaCl, 10 HEPES (pH adjusted to 7.4 with NaOH) and 5 µM tetrodotoxin. The patch pipette solution contained (in mM): 110 CsCl, 10 NaCl, 20 TEA-Cl, 14 EGTA, 20 HEPES (adjusted to pH 7.2 with CsOH) and 5 MgATP. External solutions were exchanged using a fast superfusion device consisting of a modified multi-barrelled ejection pipette [7]. The pipette had an opening of 50–100 µm and was positioned 20–50 µm away from the cell. Changes between control and test solutions were done using miniature electrovalves (The Lee Company, Westbook, Conn., USA). The flow rate (0.2–0.5 ml·min^{-1}) was regulated by gravity to achieve complete replacement of the cell surroundings within less than 1 s.

Current recordings were filtered at 3–10 kHz (–3dB, 8-pole Bessel filter) and digitized at sampling intervals of 100 µs using a 12-bit A/D Tecmar Lab Master board (125 kHz) interfaced to an IBM-compatible computer. Stimulation and acquisition of data were made with pClamp software (Axon Instruments, Foster City, Calif., USA). Off-line data analysis and curve fittings were made using pClamp and FIG PLOT software.

Cells were clamped at a holding potential of –80 mV. Step depolarizations to 0 mV from this holding potential lasted 50 ms and were applied at intervals of 10 s to minimize the run-down of Ca^{2+} currents [10]. Cells with pronounced run-downs were discarded. Capacitative transients and leakage currents were compensated electronically and by subtracting Cd^{2+}-insensitive currents, respectively. Membrane currents were always fully blocked by 200 µM Cd^{2+}, suggesting the absence of K^+ and Cl^- currents in our recordings.

Measurements of intracellular Ca^{2+} and Zn^{2+} with fura-2

For fluorescence measurements, cells were loaded with fura-2 by incubation with 4 µM fura-2/AM for about 1 h at room temperature in standard incubation medium of the following composition (in mM): NaCl 145; KCl 5; $MgCl_2$ 1; $CaCl_2$ 1; glucose 10; Na-HEPES 10, pH 7.4. The glass coverslips were then introduced into a quartz cuvette in the sample compartment of a fluorescence spectrophotometer that allowed rapid (30–300 Hz) alternation of up to six different excitation wavelengths (Cairn Research, Newnham, Sittingbourne, Kent, UK). Fluorescence emitted above 520 nm was measured and integrated at 1-s intervals. Temperature was 30°C. Perfusion of the cuvette allowed rapid (about 10 s) changes of the media bathing the cells. Excitation wavelengths used were 340, 360 and 380 nm. $[Ca^{2+}]_i$ was estimated from the ratio of the fluorescences excited at 340 and 380 nm [14]. The uptake of Zn^{2+} was estimated from the increase of the fluorescence excited at 360 nm, which is insensitive to Ca^{2+} (see Results section for details).

Statistics

Results are given as means±SEM. To estimate the IC_{50} of Zn^{2+} required to block the stimulated catecholamine secretion, or I_{Ba}, the sigmoid inhibition curves were converted into straight lines through a logit-log plotting of the ordinate values as log (y/100–y); the straight lines were determined by least-squares fitting and the intercept with the abscissa ($y = 0$) gives the IC_{50} of Zn^{2+} required to inhibit secretion or currents. The statistical significance of the differences between two means of data was calculated by the Student's t-test; the level of significance was established at $P \leq 0.05$.

Chemicals

Collagenase A was from Boehringer Mannheim (Madrid, Spain). DMEM, fetal calf serum, penicillin and streptomycin were from Gibco (Madrid, Spain). Fura-2/AM was obtained from Molecular

Probes (USA). $ZnCl_2$ was obtained from Merck. HEPES and tetrakis(2-pyridil-methyl) (TPEN) from Sigma, Spain. All other chemicals were reagent grade from Panreac, Spain.

Results

The effects of Zn^{2+} on the basal catecholamine output from perfused adrenal glands

At the beginning of each experiment, glands were perfused with Krebs-HEPES solution for 1 h to allow their equilibration with the medium. After this period, the rate of spontaneous catecholamine release amounted to 19.9±1.1 μg·30 s^{-1} ($n = 52$ glands). Zn^{2+} enhanced this rate in a concentration-dependent manner (Fig. 1). The threshold concentration was 30 μM and the maximum effect was seen at 1 mM. At 300 μM the rate of spontaneous secretion rose to 139±12.5% of the basal level, and at 1 mM to 162±12.7% of the basal level ($P<0.01$).

The time course of the increase in the rate of spontaneous catecholamine output by 1 mM Zn^{2+} is shown in Fig. 2. In the presence of extracellular Ca^{2+} (Ca_o^{2+}), the rate of secretion increased slightly over the basal level, and then started to return to basal levels after 10 min. In contrast, in the nominal absence of Ca_o^{2+}, Zn^{2+} increased the rate of secretion more clearly. The rate of secretion rose gradually to reach a peak of 67.6±15 μg·min^{-1} in 6 min ($n = 3$ glands); the secretion rate remained high for another 10 min and then started to decline gradually. However, secretion remained elevated above basal levels even after 40 min of perfusion with Zn^{2+}.

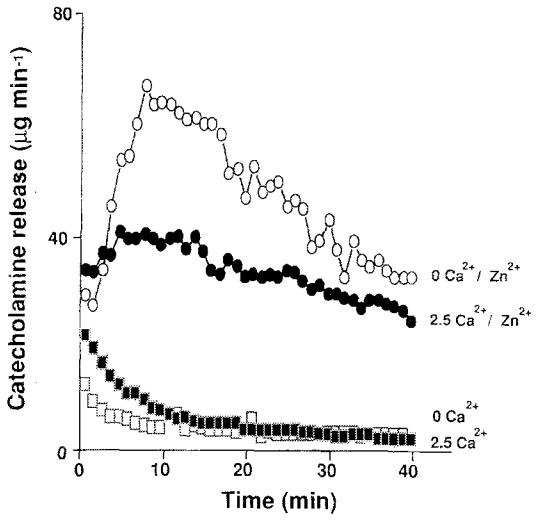

Fig. 2 Zn^{2+} increases the spontaneous release of catecholamines to a greater extent in the nominal absence of extracellular Ca^{2+} (Ca_o^{2+}). All glands were initially perfused with normal Ca^{2+}-containing Krebs-HEPES solution for 60 min. Then, some of them continued to be perfused with this solution and the others with Ca^{2+}-free Krebs-HEPES solution. Then, Zn^{2+} (1 mM) was added to Ca^{2+}-containing or Ca^{2+}-free solutions at time 0 (*abscissa*). Samples were sequentially and continuously collected at 60-s intervals. Separate glands were used for each of the four variables tested, which are represented by the *four curves* in the figure, as shown to the *right* of each *curve*. In the case of the basal secretion, data represent the means of two glands; in the case of Zn^{2+}, data are the means of three glands.

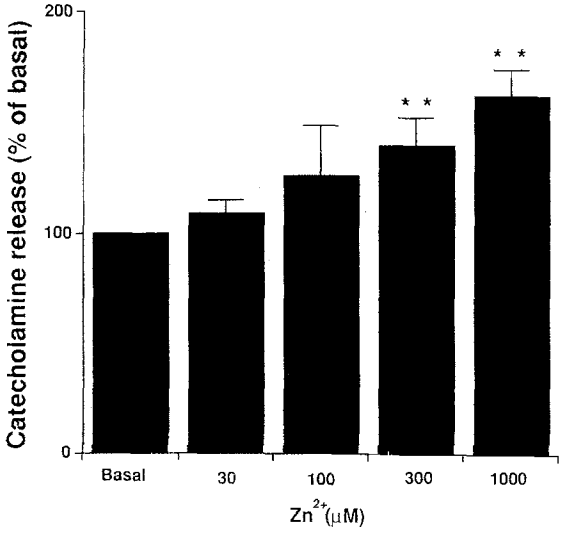

Fig. 1 Zn^{2+} increases the rate of spontaneous catecholamine release in a concentration-dependent manner. After 1 h of initial equilibration (perfusion of bovine adrenal glands with Krebs-HEPES solution, 37°C, at a rate of 15 ml·min^{-1}), the rate of basal catecholamine release was 19.9±1.1 μg·30 s^{-1} ($n = 52$). Increasing concentrations of Zn^{2+} (30–1000 μM) were then given and two 30-s samples collected 5 min after perfusion of the glands with each concentration. Data are normalized to the basal release obtained in each individual gland and are presented as a percentage. Results are means ± SEM of 9 different experiments and reflect the amount of total catecholamines collected in a 60-s period in the absence (*basal*) or after 5 min in the presence of each concentration of Zn^{2+}. $^{**}P<0.01$, compared to basal

Effects of Zn^{2+} on the stimulated catecholamine release

To evoke catecholamine release associated with depolarizing stimuli, glands were stimulated with increasing concentrations of DMPP or K^+. DMPP (10-s pulses) enhanced catecholamine release in a concentration-dependent manner; something similar occurred with K^+. Near maximal secretory responses were reached at 100 μM DMPP and 100 mM K^+. Therefore, these concentrations were selected to perform the next experiments. The secretion rate quickly increased upon DMPP stimulation (100 μM for 10 s) from the basal level of 18.9±2.2 μg·30 s^{-1} to a peak of 66.1±11 μg·30 s^{-1} ($n = 3$ glands). In the case of K^+ (100 mM for 10 s) the secretion rate rose from a basal level of 15.4±1.5 μg·30 s^{-1} to a peak of 63.4±5.8 μg·30 s^{-1} ($n = 6$ glands). After these peaks, secretion declined gradually to basal levels. When the same gland was stimulated sequentially, at 25-min intervals, with pulses of DMPP (100 μM for 10 s), the net secretion obtained with each of the seven pulses did not differ significantly from the initial secretion (148.4±30 μg; $n = 5$ glands). The same occurred when K^+ pulses (100 mM for 10 s) were applied. The net catecholamine

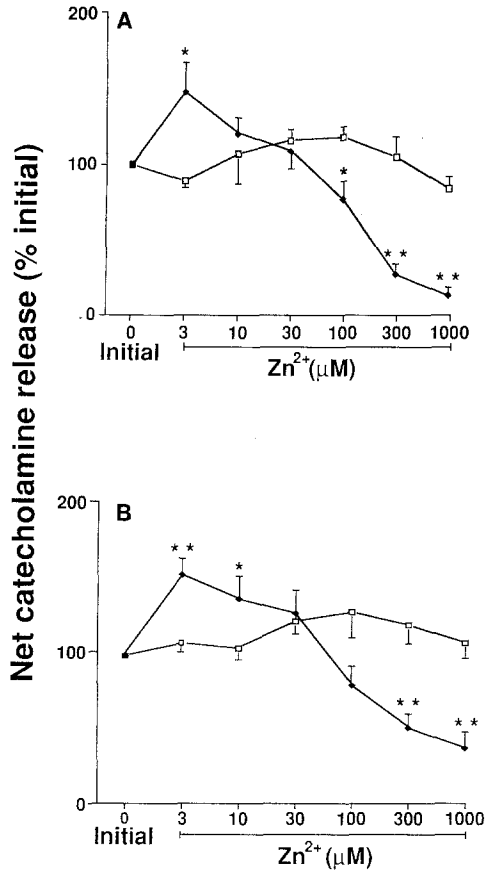

Fig. 3 Biphasic effects of Zn^{2+} on the 1,1-dimethyl-4-phenylpiperazinium (DMPP) -(**A**) and the K^+ -(**B**) evoked catecholamine release responses from perfused adrenal glands. After the initial 10-s pulse with DMPP (100 μM) or K^+ (100 mM), Zn^{2+} was introduced into the perfusion fluid at a given concentration in the absence of Ca_o^{2+}, and 5 min later the stimulating pulse was applied in the presence of 2.5 mM Ca^{2+}. Zn^{2+} was also present during the pulse and the following 50-s collection period in $0Ca^{2+}$ Krebs-HEPES solution. Each *point* reflects the net catecholamine release elicited by DMPP or K^+ in the first minute following the beginning of the pulse. Data are expressed as the % of the catecholamines recovered after an initial secreting pulse in the absence of Zn^{2+}. **A** Values are means ± SEM of 4 (*control* = □) and 5 (Zn^{2+} = ♦) glands while in **B**, they were calculated from 6 (*control*) and 8 (Zn^{2+}) glands. $^*P<0.05$, $^{**}P<0.01$ with respect to the initial release

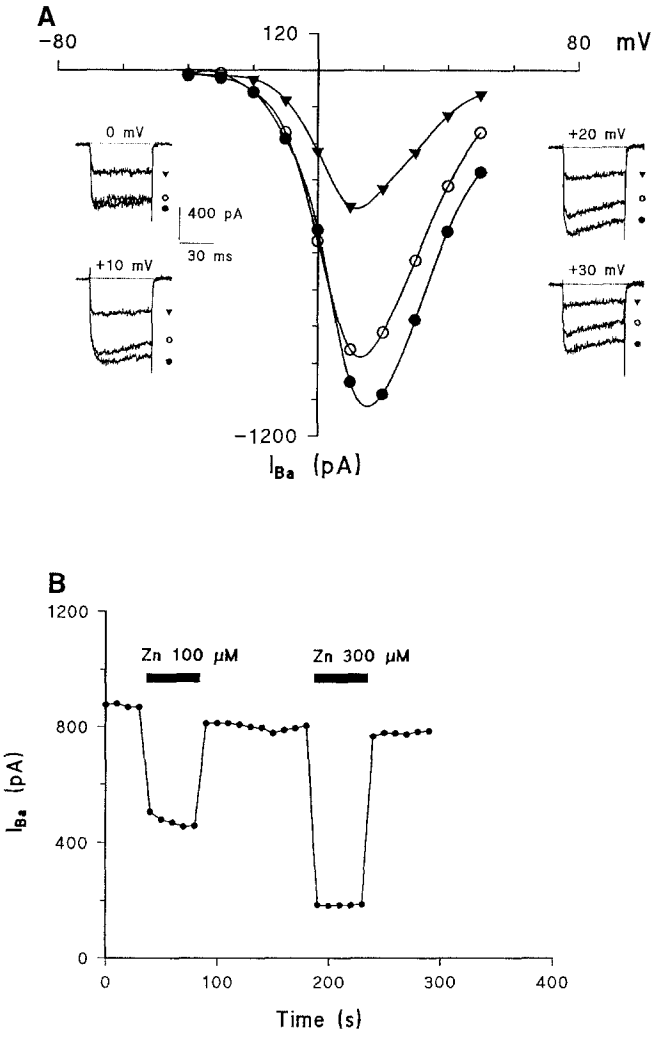

Fig. 4 Blockade by Zn^{2+} of whole-cell currents through Ca^{2+}channels. Chromaffin cells were patch-clamped in the whole-cell configuration. The holding potential was fixed at −80 mV; currents were elicited by 50-ms depolarizing pulses in 10-mV steps applied at 10-s intervals to delay the run-down of the currents. Ba^{2+} (10 mM) was the charge carrier. **A** Peak current/voltage (*I/V*) relationship obtained from a chromaffin cell before (control, ●) and after superfusion of the cell with a solution containing 30 μM (○) or 100 μM Zn^{2+} (▼). *Insets* are typical traces obtained in the absence or the presence of 30 or 100 μM Zn^{2+} at the test potentials indicated. **B** The time course and the reversibility of the blocking effects of Zn^{2+} on I_{Ba} are shown. Here, test pulses to +10 mV from a holding potential of −80 mV were applied at 15-s intervals

release obtained in pulse 1 amounted to 94.9±16 μg (n = 13 glands); in the other 6 pulses, the values obtained did not significantly differ from the initial control release. Thus, with this experimental design, a full concentration/response curve, with increasing concentrations of Zn^{2+}, could be obtained from a single gland.

Zn^{2+} modified the DMPP secretory responses following a biphasic pattern: facilitation at low concentrations and inhibition at higher concentrations. At 3 μM, Zn^{2+} increased the catecholamine release response 47±20% ($P<0.05$). At a concentration of 100 μM, or more, Zn^{2+} inhibited secretion in a concentration-dependent manner; at 1 mM, the blockade was 87±3% (Fig. 3A). The same occurred with the K^+-evoked secretory response (Fig. 3B). At 3 μM, Zn^{2+} facilitated secretion by 54±11.3%. Concentrations of Zn^{2+} higher than 100 μM blocked secretion. However, Zn^{2+} was somewhat weaker at blocking the K^+-induced secretion; 1 mM Zn^{2+} blocked the K^+ response by 63±11.5%. The IC_{50} values for the inhibition component of Zn^{2+} were 141 μM when stimulated with DMPP, and 250 μM with K^+.

Effects of Zn^{2+} on I_{Ca} and I_{Ba} through chromaffin cell Ca^{2+} channels

The dual effects of Zn^{2+} on DMPP- and K^+-evoked catecholamine release could lie in dual effects on Ca^{2+} en-

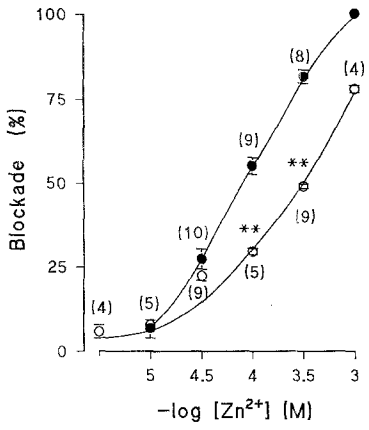

Fig. 5 Concentration/response curves for the inhibition by Zn^{2+} of I_{Ca} (○) and I_{Ba} (●) in patch-clamped chromaffin cells. See text for further details of the protocol. Data are means ± SEM with the number of cells shown *in parentheses* for each Zn^{2+} concentration. $^{**}P<0.01$ with respect to blockade of I_{Ba} at the same concentration

try through Ca^{2+} channels. To explore this possibility, whole-cell I_{Ca} and I_{Ba} currents through Ca^{2+} channels were studied in patch-clamped chromaffin cells in the whole-cell configuration. When 10 mM Ca^{2+} was present in the extracellular solution, a 50-ms test potential to 0 mV from a holding potential of −80 mV evoked I_{Ca} which averaged 670±56 pA ($n = 19$ cells). Using Ba^{2+} as the charge carrier the peak I_{Ba} rose to 817±85 pA ($n = 20$ cells), and was reached at a test potential of +10 mV.

Superfusion of bovine chromaffin cells with a solution containing increasing concentrations of Zn^{2+} led to a concentration-dependent blockade of both I_{Ca} and I_{Ba}. Figure 4A shows the control I_{Ba} (solid circles) and the effects of 30 µM (open circles) and 100 µM Zn^{2+} (solid triangles) on I_{Ba}, suggesting a non-selective blocking effect on P-, L- and N-subytpes of Ca^{2+} channels identified in these cells [1]. The blocking effects of Zn^{2+} were not accompanied by changes in the activation or inactivation kinetics of I_{Ba} (see insets to Fig. 4A). The effects of Zn^{2+} were also characterized by a fast onset of action, as well as a fast recovery upon washing out the cation from the solution (Fig. 4B).

To estimate the apparent IC_{50} values of Zn^{2+} for blocking whole-cell currents through Ca^{2+} channels, a given patch-clamped cell was sequentially exposed to various concentrations of Zn^{2+}. The effects of each Zn^{2+} concentration were estimated 2 min after superfusion of the cell with each concentration. Blocking effects were normalized with respect to the control current recorded prior to the superfusion with the Zn^{2+}-containing solution. Figure 5 shows the blocking effects of various concentrations of Zn^{2+} on both I_{Ca} and I_{Ba}. Apparent IC_{50} values were estimated to be 91 µM ($n = 4$–9 cells) and 346 µM ($n = 5$–10 cells) for I_{Ba} and I_{Ca} respectively. These values were close to those obtained for the blockade by Zn^{2+} of secretion induced by DMPP or K^+.

Permeation by Zn^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} of chromaffin cell Ca^{2+} channels

Because potentiation of I_{Ca} or I_{Ba} was never seen at the Zn^{2+} concentrations (10–30 µM) which potentiated DMPP- and K^+-evoked secretion (Fig. 4A), an alternative explanation for the facilitation by Zn^{2+} of spontaneous and evoked catecholamine release lies in an intracellular site of action. If so, Zn^{2+} must enter chromaffin cells either through Ca^{2+} channels, or through other pathways. Zn^{2+}-dependent action potentials are generated by Zn^{2+} ions permeating Ca^{2+} channels of giant snail neurones [17]. Therefore, experiments were performed in whole-cell patch-clamped chromaffin cells to investigate whether Zn^{2+} ions could carry a measurable inward current through Ca^{2+} channels, in these mammalian cells as well.

In this series of experiments, the relative permeabilities of Ca^{2+} channels to Zn^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} were explored. A given cell was sequentially superfused with 10 mM of each cation. Typical traces obtained after 2 min of superfusion, using test pulses of 0 mV from a holding potential of −80 mV, are shown in Fig. 6A. Ba^{2+} was the most permeable cation, followed by Ca^{2+} and Sr^{2+}. In the presence of 10 mM Zn^{2+}, a small inward current was generated. Peak currents were obtained at +10

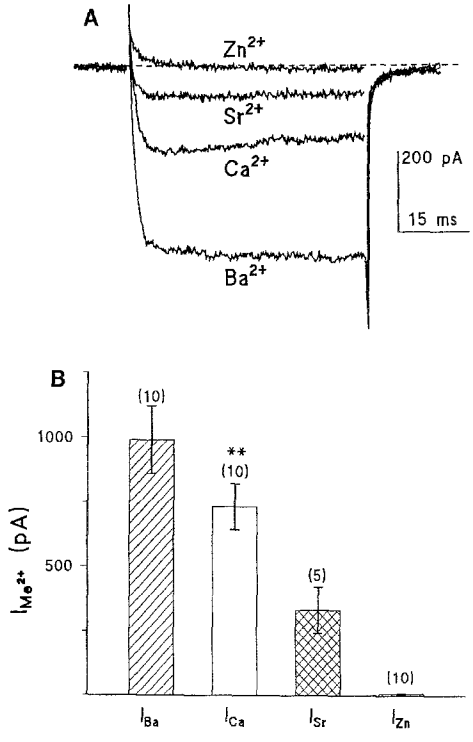

Fig. 6 Relative permeability of bovine chromaffin cell Ca^{2+} channels to Ca^{2+}, Ba^{2+}, Sr^{2+} and Zn^{2+}. **A** Original records obtained from a chromaffin cell by using 10 mM of each cation as the charge carrier. Each record was obtained after a 2-min superfusion with a solution containing the corresponding cation. **B** Averaged peak currents ($I_{Me^{2+}}$) obtained from 5–10 cells. $^{**}P<0.01$ with respect to I_{Ba}

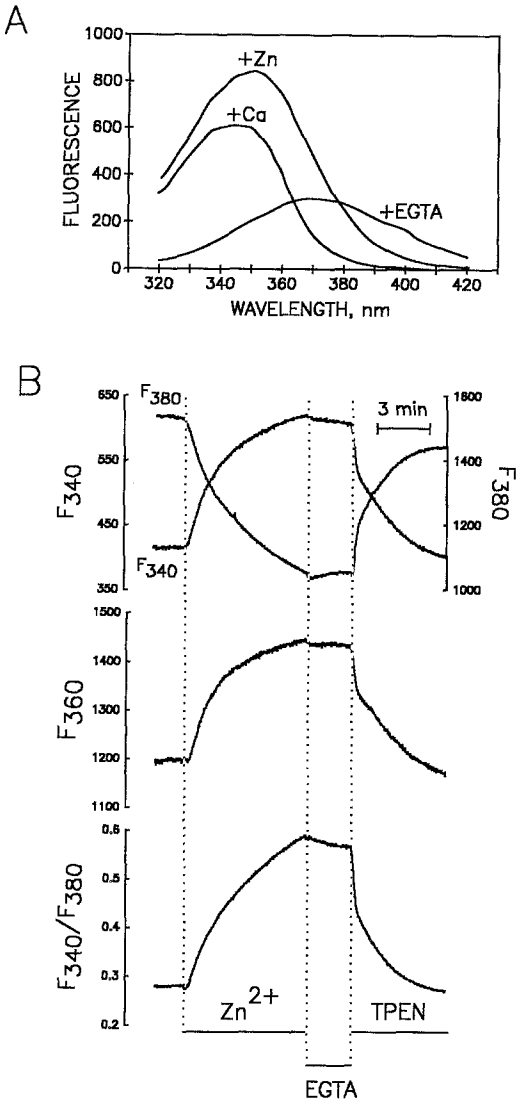

Fig. 7 **A** Excitation spectra for free fura-2 (*+EGTA*) and its complexes with Ca^{2+} (*+Ca*) and Zn^{2+} (*+Zn*). **B** Entry of Zn^{2+} into non-stimulated fura-2-loaded bovine chromaffin cells. Effects on the fluorescences excited at 340, 380 and 360 nm are shown. The incubation medium was nominally free of Ca^{2+}. The additions to the perfusing solution are indicated below the *traces*. The concentrations used were (in mM): Zn^{2+}, 1; EGTA, 1; tetrakis(2-pyridilmethyl) (TPEN), 0.03. Experiment representative of four similar ones

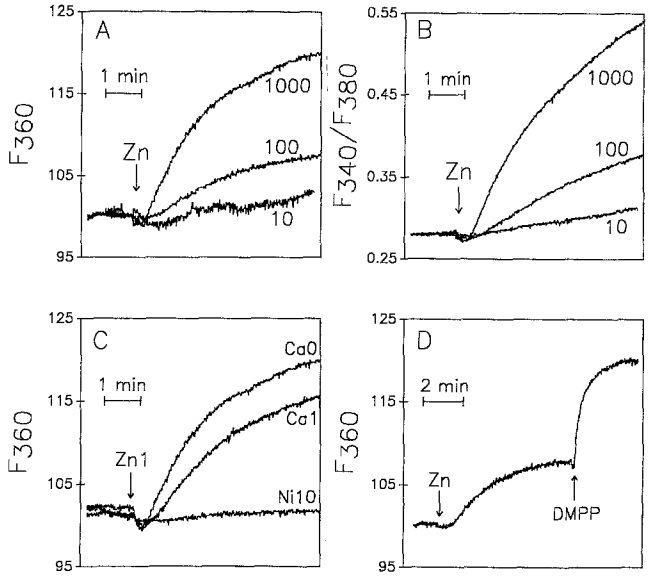

Fig. 8 Entry of Zn^{2+} into non-stimulated bovine chromaffin cells and stimulation by DMPP. **A, B** Concentration dependence in cells incubated in Ca^{2+}-free medium; Zn^{2+} entry is given as assessed by the fluorescence emitted by excitation at 360 nm F_{360} **(A)** or as F_{340}/F_{380} **(B)**. Zn^{2+} was added at the time shown by the *arrow* at a concentration of either 10, 100 or 1000 μM as shown. **C** Effects of external Ca^{2+} (1 mM) and Ni^{2+} (10 mM). Zn^{2+} (1 mM) was added at the time shown by the *arrow*. **D** Zn^{2+} (100 μM) and DMPP (5 μM) were added at the times shown by the *arrows*. Experiment representative of three similar ones

mV with Ba^{2+}, −10 mV with Ca^{2+} and +10 mV with Sr^{2+}. Small inward currents were observed at 0 mV when 10 mM Zn^{2+} was used as the charge carrier. Averaged peak currents for each cation obtained in 5–10 different cells are shown in Fig. 6B. The peak I_{Ba} was 991±130 pA, I_{Ca} amounted to 734±88 pA, I_{Sr} to 330±88 pA and I_{Zn} amounted to 7±2 pA.

Measurements of Zn^{2+} entry into fura-2-loaded chromaffin cells

We thought that fura-2 fluorescence would be more sensitive for measuring Zn^{2+} fluxes than patch-clamp recording of currents. In fact, fura-2 has been recently used to measure free nanomolar concentrations of Zn^{2+} in nuclei isolated from bovine liver [16]; the K_d value for the fura-2-Zn^{2+} complex was estimated to be about 0.5 nM [2], 2 orders of magnitude below the K_d for Ca^{2+}. On the other hand, Zn^{2+} displaced the excitation spectrum of fura-2 towards longer wavelengths, as does Ca^{2+}, but the isosbestic points for both cations differed (Fig. 7A). At 360 nm excitation, the isosbestic point for Ca^{2+}, Zn^{2+} increased the fluorescence of fura-2. Therefore, the increase of the fluorescence excited at this wavelength does not depend on $[Ca^{2+}]_i$, but reflects Zn^{2+} entry into the cells. This procedure is similar to the one reported before for measuring Ba^{2+} entry [2].

Figure 7B illustrates Zn^{2+} entry into fura-2-loaded chromaffin cells. Addition of 1 mM Zn^{2+} to cells incubated in Ca^{2+}-free medium was followed by an increase of fluorescence at 340 nm (F_{340}) and a decrease of F_{380} (upper traces). These changes must reflect entry of Zn^{2+} into the cells, since the incubation medium contained no Ca^{2+}. The simultaneous increase of F_{360} (middle trace), the Ca^{2+}-insensitive wavelength, confirmed this view. The increase in fura-2 fluorescence excited at 360 nm is therefore due to Zn^{2+} entry and not to leakage of the dye. Excess EGTA was added to determine whether the increase in fura-2 fluorescence excited at 360 nm was due to Zn^{2+} entry, or some kind of artefact and/or leakage of the dye. Thus, in the absence of external Zn^{2+} (EGTA-containing medium), there was not a decrease of the flu-

orescence generated by Zn^{2+} entry prior to the addition of EGTA. This suggests that Zn^{2+} is not a substrate for the Ca^{2+} pump and/or the Na^+/Ca^{2+} exchanger, and remained accumulated inside the cells. On the contrary, perfusion with TPEN, a permeant Zn^{2+} chelator, produced a switch of fluorescence to its original values. These results corroborate the view that the increase of F_{360} was due to Zn^{2+} located inside the cells. The lower trace in Fig. 7B shows that the ratio of the fluorescences F_{340}/F_{380} increased with Zn^{2+}, the percentage change being larger than that observed for F_{360}. Under the conditions of the experiment, with no Ca^{2+} present, changes in this ratio are a good measurement of Zn^{2+} entry. However, when Ca^{2+} is present, variations of F_{340}/F_{380} do not distinguish between changes of either $[Ca^{2+}]_i$ or $[Zn^{2+}]_i$ and estimations of Zn^{2+} entry must rely only on measurements of F_{360}.

Figure 8 illustrates the properties of Zn^{2+} entry in non-stimulated chromaffin cells. A concentration-dependent Zn^{2+} entry was evidenced in Ca^{2+}-free medium by the increase of either F_{360} (A), or the ratios F_{340}/F_{380} (B). Zn^{2+} entry (tested at 1 mM) was decreased by the simultaneous presence of 1 mM Ca^{2+} and fully blocked by 10 mM Ni^{2+} (C). At 5 mM Ni^{2+}, the blockade of Zn^{2+} entry was also complete (results not shown). The entry of Zn^{2+} (100 μM) was stimulated by DMPP (D).

The idea that Zn^{2+} enters the cells through voltage-dependent channels was supported by the finding that depolarization with high-K^+ solutions also accelerated the entry of Zn^{2+}. This is illustrated in the experiment of Fig. 9 where chromaffin cells were stimulated with high-K^+ solution (containing also 1 mM Zn^{2+}) either in the absence of Ca^{2+}, in medium containing 1 mM Ca^{2+}, or in medium containing 1 mM Ca^{2+} and 10 mM Ni^{2+}. The entry of Zn^{2+} in non-stimulated cells is also shown for comparison (dotted trace). The entry of Zn^{2+} in K^+-stimulated cells was decreased by Ca^{2+}, and fully blocked by Ni^{2+}.

Next, we attempted to investigate whether the presence of Zn^{2+} interfered with Ca^{2+} entry in stimulated cells. Figure 10 shows simultaneous measurements of F_{340}/F_{380} (upper panels) and F_{360} (lower panels) in cells stimulated either with high-K^+ solution (left) or DMPP (right). The incubation medium always contained 1 mM Ca^{2+}. Stimulation was performed either in the presence (+Zn) or in the absence (control) of 1 mM Zn^{2+}. The traces at F_{360} illustrate the entry of Zn^{2+} in stimulated cells and the lack of effect of Ca^{2+} entry at this fluorescence (control). The ratio F_{340}/F_{380} increased with both Ca^{2+} and Zn^{2+}, although the increase was larger with Ca^{2+}. Theoretically, the values of F_{340} and F_{380} could be corrected to subtract the effect of Zn^{2+}, estimated from variations of F_{360}, and thus obtain a corrected value of F_{340}/F_{380}, only representative for Ca^{2+}. This approach proved to be unreliable in practice, however, the faster kinetics of Ca^{2+} entry allowed some semiquantitative estimates. The peak values of F_{340}/F_{380} were obtained

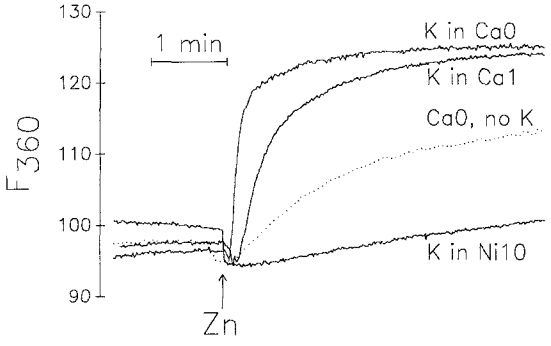

Fig. 9 Acceleration of Zn^{2+} entry by depolarization with high-K^+ solution. Zn^{2+} (1 mM) was added at the time shown by the *arrow* in high-K^+ solution (50 mM) containing either no Ca^{2+}, 1 mM Ca^{2+} or 1 mM Ca^{2+} and 10 mM Ni^{2+}, as shown. Entry of Zn^{2+} in cells incubated in low-K^+ (5 mM) medium is shown by the *dotted line*. Experiment representative of three similar ones

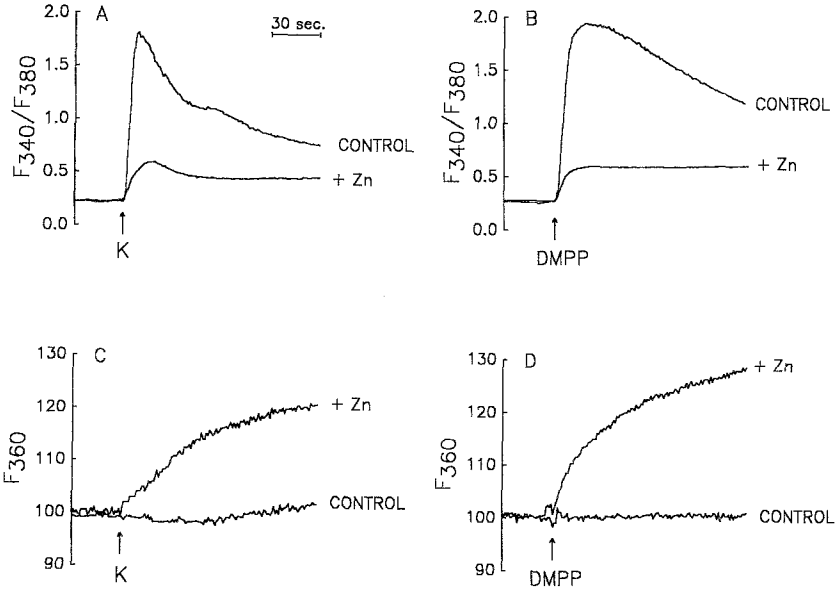

Fig. 10 Effects of Zn^{2+} (1 mM) on the entry of Ca^{2+} in cells stimulated by high-K^+ solution (50 mM, *left*) or by DMPP (5 μM, *right*). The incubation medium always contained 1 mM Ca^{2+}. When used, Zn^{2+} was 1 mM. Stimulants were added as shown by *arrows*. The *upper panels* show the ratio between the fluorescences excited at 340 and 380 nm. The *lower panels* show the fluorescence excited at 360 nm, measured simultaneously. The experiments with high-K^+ solution (**A,C**) and DMPP (**B,D**) were performed in different cell batches. The effect of high-K^+ solution on Zn^{2+} entry was usually somewhat greater than that of DMPP. Experiment representative of three similar ones for high-K^+ solution and two similar ones for DMPP

10–15 s after stimulation in control cells. At these times, the entry of Zn^{2+} into the cells stimulated in the presence of this cation was less than 20% of the final value, suggesting that occupation of fura-2 by Zn^{2+} should be similarly low. Hence, the ratio F_{340}/F_{380} at these short times should reflect mainly $[Ca^{2+}]_i$ levels. The figure shows that peak F_{340}/F_{380} values at 10–15 s were strongly depressed by Zn^{2+} in both K^+-stimulated (A) and DMPP-stimulated (B) cells, suggesting that Ca^{2+} entry was inhibited by Zn^{2+}. This is consistent with the inhibition through voltage-dependent Ca^{2+} channels of Ca^{2+} entry by Zn^{2+} as demonstrated by patch-clamp current measurements (see above). At latter times, F_{340}/F_{380} approached a value of 0.5, characteristic of the fura-2-Zn^{2+} complex, evidence of Zn^{2+} entry.

Discussion

The various effects of Zn^{2+} on catecholamine release from the perfused bovine adrenal gland are as follows: (1) Zn^{2+} increased the rate of spontaneous catecholamine secretion; (2) in the absence of Ca_o^{2+}, Zn^{2+} was more potent as a secretagogue than in its presence; (3) at low concentrations, Zn^{2+} facilitated the DMPP- as well as the K^+-evoked secretory responses; (4) at higher concentrations, Zn^{2+} blocked the stimulated catecholamine release.

The increase of the spontaneous rate of secretion produced by Zn^{2+} can be best explained if Zn^{2+} enters chromaffin cells, as it does in neurones [26]. The slow development of the Zn^{2+} secretory actions is a second argument in favour of a time-dependent intracellular accumulation of Zn^{2+}. It is curious that Zn^{2+} is more potent in exerting these effects in the absence of Ca_o^{2+}, thus suggesting that Zn^{2+} enters chromaffin cells in competition with Ca^{2+} for a common binding site on the plasmalemma. This is corroborated by the greater Zn^{2+} entry (measured in fura-2-loaded cells) in the absence of Ca_o^{2+}. Zn^{2+} could trigger the secretion of catecholamines either by activating the secretory machinery directly, or through the release of Ca^{2+} from intracellular stores. A third mechanism could be involved since Zn^{2+} has been shown to activate protein kinase C (PKC) [8], which is known to increase the sensitivity for Ca^{2+} of the secretory machinery in chromaffin cells [18].

A puzzling observation was the facilitation by low concentrations of Zn^{2+} of the nicotinic-mediated secretory response. The modulatory effects of Zn^{2+} on the ionic permeability of the NMDA-receptor-associated channel in central neurones is well established [20]. Also Zn^{2+} has been shown to block pre-synaptic $GABA_B$ receptors, thus enhancing the release of GABA in rat hippocampal slices [28]. The facilitation by Zn^{2+} of the DMPP-triggered secretion could be also due to a direct action of Zn^{2+} on the nicotinic-receptor-associated ionophore. However, though this action can not be discarded, the facilitation of the K^+-evoked response suggests that, if acting on the nicotinic receptor, its effects are not exclusively located at this site. So, the facilitation of Ca^{2+} entry through voltage-dependent Ca^{2+} channels could be responsible for the facilitation of secretion. However, the experimental evidence obtained was in favour of a blocking, rather than a facilitatory, effect.

Concerning Ca^{2+} channels, it is known that Zn^{2+} is capable of carrying currents through them in invertebrate neurones, and of generating Zn^{2+} action potentials [17]. In addition, Zn^{2+} blocks voltage-gated Ca^{2+} channels in dorsal root ganglion cells [6], *Aplysia* neurones [5] and mouse myotubes [27]. Here, we have shown that Zn^{2+} also inhibited the I_{Ca} and I_{Ba} through chromaffin cell Ca^{2+} channels in a concentration-dependent manner. Therefore, the blockade of DMPP- and K^+-evoked secretion at the higher concentrations of Zn^{2+} might be due to inhibition of the voltage-dependent Ca^{2+} channels recruited by those depolarizing agents. Conversely, the facilitation could be explained if low concentrations of Zn^{2+} enhance Ca^{2+} entry through those channels. However, at concentrations that potentiated secretion, Zn^{2+} did not enhance the whole-cell I_{Ca} or I_{Ba}; on the contrary, Zn^{2+} decreased the current.

Nevertheless, current measurements demonstrated small but significant permeation of Zn^{2+} through Ca^{2+} channels. Measurements with fura-2 also indicated Zn^{2+} entry, which was accelerated by stimulation by DMPP or by high-K^+ solutions, and blocked by Ni^{2+}. The conditions established here for measurements of Zn^{2+} entry with fura-2 in chromaffin cells likely can be extrapolated to other cell kinds, particularly to neurones, where Zn^{2+} has been proposed to play a role as a synaptic regulator.

The facilitation by Zn^{2+} of catecholamine release may then be due to an intracellular action. The behaviour of Zn^{2+} closely reminds that of Pb^{2+} in permeabilized bovine chromaffin cells [25]. In these cells, Pb^{2+} activated noradrenaline release at considerably lower concentrations ($K_{0.5}$, 4.6 nM) than Ca^{2+} ($K_{0.5}$, 2.4 μM). Such concentrations of Pb^{2+} are in the range of the concentrations likely to have been reached by Zn^{2+} in our present experimental conditions, since the K_D for the Zn^{2+}-fura-2 complex was estimated to be about 0.5 nM [2]. This implies that Zn^{2+}-induced changes of fura-2 fluorescence should take place within this concentration range. Tomsig and Suszkiw [25] observed that Pb^{2+}- and Ca^{2+}-induced releases were similarly enhanced by activation of PKC and inhibited by calmodulin blockade. Thus, they concluded that Pb^{2+} and Ca^{2+} act at a common site and activate the exocytotic release of catecholamines by an analogous mechanism. A priori, this also could be the mechanism involved in the secretory effects of Zn^{2+}. Contrary to Pb^{2+} that has only toxicological interest, Zn^{2+} has been implicated in the physiological regulation of several aspects of synaptic neurotransmission [29]. Therefore, the clarification of its intracellular site(s) of action in permeabilized cells, or through the dialysis of Zn^{2+} via a patch-clamp pipette deserves further consideration.

In conclusion, we demonstrate here that Zn^{2+} exerts dual effects on the bovine chromaffin cell secretory machinery, causing potentiation or inhibition of secretion. Inhibition of evoked secretion seems to be associated

with its well established actions as an inorganic Ca^{2+} channel blocker. Facilitation of exocytosis, however, seems to be exerted at an intracelullar site on the secretory machinery. This is plausible because, as demonstrated in fura-2-loaded chromaffin cells, Zn^{2+} permeates Ca^{2+} channels and gains access to the cytosol through them. In addition, we have established the conditions needed to estimate Zn^{2+} transients in fura-2-loaded cells at nanomolar concentrations of Zn^{2+}. In view of the increasing importance of Zn^{2+} as a synaptic regulator, this methodology can be useful for defining Zn^{2+} signals in various neuronal cell types.

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