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Effect of Gd^{3+} on bradykinin-induced catecholamine secretion from bovine adrenal chromaffin cells

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1 The effects of Gd^{3+} on bradykinin- (BK-) induced catecholamine secretion, $^{45}Ca^{2+}$ efflux and cytosolic $[Ca^{2+}]$ were studied using bovine adrenal chromaffin cells.

2 BK increased secretion in a Ca^{2+}-dependent manner. From 1–100 μM, Gd^{3+} progressively inhibited secretion induced by 30 nM BK to near-basal levels, however from 0.3–3 mM Gd^{3+} dramatically enhanced BK-induced secretion to above control levels. Gd^{3+} also increased basal catecholamine secretion by 2–3 fold at 1 mM. These effects were mimicked by Eu^{3+} and La^{3+}.

3 Gd^{3+} enhanced secretion induced by other agonists that mobilize intracellular Ca^{2+} stores, but simply blocked the response to K^+.

4 Gd^{3+} still enhanced basal and BK-induced secretion in Ca^{2+}-free solution or in the presence of 30 μM SKF96365, however both effects of Gd^{3+} were abolished after depleting intracellular Ca^{2+} stores.

5 Gd^{3+} (1 mM) reduced the rate of basal $^{45}Ca^{2+}$ efflux by 57%. In Ca^{2+}-free buffer, BK transiently increased cytosolic $[Ca^{2+}]$ measured with Fura-2. The $[Ca^{2+}]$ response to BK was substantially prolonged in the presence of Gd^{3+} (1 mM).

6 The results suggest that Gd^{3+} greatly enhances the efficacy of Ca^{2+} released from intracellular stores in evoking catecholamine secretion, by inhibiting Ca^{2+} extrusion from the cytosol. This suggests that intracellular Ca^{2+} stores are fully competent to support secretion in chromaffin cells to levels comparable to those evoked by extracellular Ca^{2+} entry. Drugs that modify Ca^{2+} extrusion from the cell, such as lanthanide ions, will be useful in investigating the mechanisms by which intracellular Ca^{2+}-store mobilization couples to Ca^{2+}-dependent exocytosis.

Keywords: Ca^{2+} channels; chromaffin cells; catecholamine secretion; Gd^{3+}; Na^+/Ca^{2+}-exchanger; Ca^{2+}-ATPase; Ca^{2+} stores; inositol-1,4,5-trisphosphate

Abbreviations: BK, bradykinin; HBS, HEPES-buffered solution; h.p.l.c., high pressure liquid chromatography; $Ins(1,4,5)P_3$, inositol-1,4,5-trisphosphate; SOC, store-operated channel; VOCC, voltage-operated Ca^{2+} channel

Introduction

Many G protein-coupled receptors stimulate Ca^{2+} release from intracellular stores. At least two types of store are present in adrenal chromaffin cells, one mobilized by inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and another by the ryanodine receptor agonist caffeine (Liu *et al*., 1991; Robinson & Burgoyne, 1991; see Cheek & Barry, 1993). Ca^{2+} mobilization from the $Ins(1,4,5)P_3$-sensitive store occurs in a fairly restricted region of the cell, while caffeine-sensitive stores are evenly distributed throughout the cytoplasm (Cheek & Barry, 1993). There may be some functional overlap between the two stores, since release of Ca^{2+} from both is influenced by cytosolic Ca^{2+} levels. Ca^{2+} can be released from both types of store by receptor stimulation (see Tanaka *et al*., 1998).

Mobilization of Ca^{2+} from intracellular stores in chromaffin cells, either by $Ins(1,4,5)P_3$-generating agonists or by caffeine, results in the activation of store-operated channels (SOCs) in the plasma membrane (Liu *et al*., 1991; Cheek & Barry, 1993). These gate the entry of extracellular Ca^{2+} in the process of capacitative Ca^{2+} entry. Although the mechanism by which SOCs are activated is yet to be determined (Parekh & Penner, 1997; Barritt, 1999), it is clear that mobilization of Ca^{2+} stores *per se* with caffeine or thapsigargin is an adequate stimulus to induce capacitative Ca^{2+} entry in these cells (Liu *et al*., 1991; Robinson *et al*., 1992; Zerbes *et al*., 1998). Activation of G proteins or second messenger formation by receptors is not essential for this process.

The role of Ca^{2+} entry through SOCs in chromaffin cells is poorly understood. It contributes to elevations of cytosolic Ca^{2+} and possibly to Ca^{2+} oscillations, but the purpose of these complex signals is unclear (Cheek & Barry, 1993; D'Andrea *et al*., 1993; Finnegan *et al*., 1996). Mobilization of store Ca^{2+} contributes to the phosphorylation of tyrosine hydroxylase, the rate-limiting enzyme for catecholamine biosynthesis (Haycock, 1993), and to a small increase in catecholamine secretion (Cheek & Thastrup, 1989; Powis *et al*., 1996). One possible function of SOCs is to depolarize the cell and thereby recruit voltage-operated Ca^{2+} channels (VOCCs; see Barritt, 1999). Inhibitors of SOCs, including SKF96365 and lanthanide ions such as Gd^{3+}, Eu^{3+} and La^{3+}, have been successfully used to investigate their function in non-excitable cells (e.g., Demaurex *et al*., 1992; Fatatis *et al*., 1992; Fernando & Barritt, 1994; 1995; Zimmermann, 1998). Their use in excitable cells such as chromaffin cells, however, is complicated by their non-selective effects on other Ca^{2+}-permeant channels including VOCCs and receptor-operated channels (Tachikawa *et al*., 1994).

In spite of this, in the present study we have used a lanthanide ion, gadolinium (Gd^{3+}), to investigate the role of extracellular Ca^{2+} in BK-stimulated catecholamine secretion in bovine chromaffin cells. BK evokes secretion though B2

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receptors and this response is largely or fully dependent on extracellular Ca^{2+} (Kim & Westhead, 1989; O'Sullivan & Burgoyne, 1989; Owen *et al*., 1989b; Finnegan & Wightman, 1995; Kim & Kim, 1998). BK increases Ins(1,4,5)P_3 formation and mobilizes intracellular Ca^{2+} from both caffeine- and Ins(1,4,5)P_3-sensitive stores (Plevin & Boarder, 1988; O'Sullivan & Burgoyne, 1989; Challiss *et al*., 1991; Castro *et al*., 1995; Tanaka *et al*., 1998). It also causes a sustained influx of extracellular Ca^{2+} (O'Sullivan & Burgoyne, 1989; Castro *et al*., 1995), however the secretory response to BK is unaffected by antagonists of L- or N-type VOCCs (Owen *et al*., 1989a,b; McMillian *et al*., 1992). Since it mobilizes intracellular Ca^{2+} stores, it is likely that at least part of BK's secretory response depends on Ca^{2+} entry through SOCs.

We therefore investigated the effects of Gd^{3+}, a known inhibitor of SOCs in other cell types (see above), on BK-induced catecholamine secretion from bovine chromaffin cells. Unexpectedly, we found high concentrations of Gd^{3+} enhanced BK-induced secretion. Consequently, this effect was investigated further. Preliminary reports of this work have been published (Bales & Marley, 1998; Marley *et al*., 1999).

Methods

Catecholamine release from bovine adrenal chromaffin cells

Catecholamine secretion and $^{45}Ca^{2+}$ efflux were studied using primary cultures of bovine adrenal chromaffin cells prepared as described previously (Livett *et al*., 1987b). Cells were cultured at a density of 0.5×10^6 cells/well in 24-well plates for 3–6 days before use. For catecholamine release, culture plates were placed on a 37°C warming tray and given a series of treatments in pre-warmed HEPES-buffered solution (HBS) (in mM: NaCl 150, KCl 2.6, $MgCl_2$ 1.18, $CaCl_2$ 2.2, D-glucose 10, HEPES 10, pH was adjusted to 7.4 with NaOH). Solutions containing phosphate, bicarbonate, sulphate or Tris were not used because of their tendency to form complexes with lanthanide ions (see Caldwell *et al*., 1998). Bovine serum albumin was omitted from all buffers, as a precipitate formed in solutions containing albumin and mM concentrations of certain heavy metals ions. The omission of albumin resulted in higher basal rates of catecholamine secretion.

Before any treatments (see below), all cells were given two 10 min washes in HBS. Catecholamine release was measured over a 10 min stimulation period in HBS containing BK, high K^+ concentrations or other agonists as stated. At the end of the 10 min stimulation period, the supernatant was removed and acidified with perchloric acid to a final concentration of 0.4 M, and the remaining cellular catecholamines were extracted also into 0.4 M perchloric acid. Catecholamines were measured in the samples by h.p.l.c. with electrochemical detection essentially as described by Livett *et al*. (1987a), except that the h.p.l.c. mobile phase comprised 70 mM KH_2PO_4, 0.5 mM Na_2EDTA, 8 μM sodium octane sulphonate, pH adjusted to 3.0 with orthophosphoric acid, and 12% methanol. The sensitivity of the electrochemical detector to catecholamines was unaffected by the presence of up to 10 mM Gd^{3+} in the samples.

In experiments assessing the effects of Gd^{3+} and other heavy metals, cells received a 15 min pre-incubation with the metal ions, before the 10 min stimulation period with agonist in the continued presence of metal ions. Similarly, SKF96365 was included in a 15 min pre-incubation as well as the 10 min stimulation with agonist. High K^+ concentrations used to depolarize the cells were made by adding the stated additional concentration of KCl to the HBS with equimolar removal of NaCl. Effects of Ca^{2+}-free HBS were determined by omitting $CaCl_2$ from the HBS for a 15 min pre-incubation as well as throughout the 10 min stimulation period. Depletion of intracellular Ca^{2+} stores was achieved by pre-treating the cells for 30 min in Ca^{2+}-free HBS in the presence of either 10 mM caffeine and 10 μM ryanodine or 100 nM thapsigargin. The cells then received a 15 min pre-incubation in the same conditions together with heavy metal ions, before the 10 min stimulation period with agonist in the continued presence of store-depleting drugs and heavy metal ions, all in Ca^{2+}-free HBS.

$^{45}Ca^{2+}$ efflux

$^{45}Ca^{2+}$ efflux from chromaffin cells was determined by adapting the technique described by Houchi *et al*. (1994). Cells were prepared and plated as for catecholamine release experiments. After 3–5 days in culture, the culture medium was removed and cells were loaded overnight (approximately 18 h) in 0.6 ml culture medium without foetal calf serum with 3 μCi $^{45}Ca^{2+}$ ml^{-1} (final specific activity: 2.86 μCi μmol^{-1}). The following day, plates were placed on a 37°C warming tray. The cells then received a total of 20 successive incubations in 0.8 ml pre-warmed HBS. First, they received eight 1 min washes in HBS which were discarded. Then basal $^{45}Ca^{2+}$ efflux was measured over four 1 min periods. The cells then received eight 1 min treatments with HBS with or without Gd^{3+}. The remaining cellular $^{45}Ca^{2+}$ was extracted by lysing the cells in 0.4 M perchloric acid. $^{45}Ca^{2+}$ content of the release samples and the cell extracts were analysed by liquid scintillation counting.

Measurement of cytosolic [Ca^{2+}] with Fura-2

Measurements of cytosolic [Ca^{2+}] with Fura-2 were performed using chromaffin cells prepared and cultured on glass cover slips as described previously (Powis *et al*., 1996; Zerbes *et al*., 1998). Cells were loaded with Fura-2 by incubation for 60 min at ambient temperature in HBS containing Fura-2-acetoxymethyl ester (2 μM), Pluronic F-127 (0.2 mg ml^{-1}) and bovine serum albumin (0.1% w v^{-1}). After loading, cells were incubated in albumin-containing HBS (0.2% w v^{-1}) for 60 min, to allow complete de-esterification of the indicator, and then exposed for 30 min to Ca^{2+}-free HBS with or without thapsigargin (100 nM). Coverslips were then mounted in a cuvette and continuously perfused with Ca^{2+}-free HBS at approximately 22°C. Fluorescence (excitation: 340 and 380 nm; emission: 510 nm) was measured throughout with a Perkin-Elmer LS50B spectrofluorimeter. Fluorescence ratios (340 nm/380 nm) were calculated after correcting for autofluorescence as described previously (Zerbes *et al*., 1998). Fluorescence was measured for 5 min to establish baseline cytosolic [Ca^{2+}] before the perfusion solution was switched for 15 min to Ca^{2+}-free HBS with or without Gd^{3+} (1 mM). The cells were then perfused for 10 min with Ca^{2+}-free HBS containing BK (100 nM) in the continued presence of Gd^{3+} if used.

Data presentation and statistics

Catecholamine secretion is expressed as a percentage of cellular catecholamine content at the start of the stimulation period that was secreted during the 10 min stimulation period. $^{45}Ca^{2+}$ efflux is expressed as a fraction (%) of the cellular $^{45}Ca^{2+}$ content at the start of that 1 min period. Data are presented in the figures as mean ± s.e.mean for the stated

number of observations from a single cell preparation and are representative of similar results on the stated number of cell preparations. Where error bars are not visible, they lie within the size of the symbol. Values given in the text are the range of effects seen in the stated number of cell preparations. Fura-2 data are presented as fluorescence ratios (340 nm/380 nm), with representative traces from three cell preparations being shown for each treatment condition.

For statistical comparisons between two treatment groups, unpaired Student's *t*-test was used. For multiple comparisons, the results were analysed by analysis of variance with appropriate *post hoc* tests: Dunnett's test for multiple comparisons against a single control group, and the Tukey-Kramer test for multiple comparisons. Interaction between treatments was assessed as described previously (Moeller *et al*., 1989). The methods of analysis are identified in the figure legends. Only selected statistical comparisons are shown for clarity.

Materials

Cadmium chloride, caffeine, cobalt chloride, gadolinium chloride, histamine dihydrochloride and strontium chloride were from Sigma Chemical Co. (U.S.A.). Bradykinin was from Auspep Pty. Ltd. (Australia). Angiotensin II was from Peninsula Lab (U.S.A.). Europium chloride was from Aldrich, nickel chloride from FSE and lanthanum chloride from BDH Laboratories. Fura-2-acetoxylmethyl ester and Pluronic F-127 were from Molecular Probes (Portland, OR, U.S.A.). $^{45}Ca^{2+}$ ($^{45}CaCl_2$ in aqueous solution, specific activity 19 mCi mg^{-1} calcium) was from Amersham Life Sciences (Australia). Thapsigargin and ryanodine were from Research Biochemicals International (U.S.A.). SKF96365 was from Calbiochem (Germany). Thapsigargin, ryanodine, SKF96365, Fura 2 and Pluronic F-127 were each dissolved in dimethylsulphoxide before dilution in buffer. Appropriate solvent controls were always performed.

Results

BK-induced catecholamine secretion

BK produced a concentration-dependent increase in secretion of both adrenaline and noradrenaline from bovine adrenal chromaffin cells (data not shown). The EC_{50} was between 0.1 and 1 nM and the response approached maximum at 10–30 nM. With 30 nM BK, noradrenaline (NA) secretion amounted to 5.4 ($\pm$0.5)% and adrenaline (Adr) secretion amounted to 2.4 ($\pm$0.2)% cell content released in 10 min, respectively (mean$\pm$s.e.mean of effects from 19 cell preparations: range of effects – NA 2.3–11.4% 10 min^{-1}, Adr 1.2–5.9% 10 min^{-1}). BK was therefore substantially more effective at evoking NA release than Adr release, as has been reported by others (Owen *et al*., 1989b). Since the Adr responses were very small, only NA responses are presented for the remaining experiments for the sake of clarity, although qualitatively similar results were obtained for Adr.

In most experiments, the secretory response to 30 nM BK was dependent on extracellular Ca^{2+}: BK was unable to evoke secretion in Ca^{2+}-free buffer in 12 of 17 experiments (see Figures 3a, and 5a,b). This is in agreement with previous studies which found BK-induced-catecholamine secretion to be mostly (O'Sullivan & Burgoyne, 1989; Owen *et al*., 1989b) or completely dependent on extracellular Ca^{2+} (Kim & Westhead, 1989).

Gd^{3+} effects on NA secretion

The effects of Gd^{3+} were tested on BK-induced catecholamine secretion, since this cation is a known inhibitor of SOCs in other cell types (see Introduction). Gd^{3+} produced a significant, concentration-dependent enhancement of basal NA secretion (Figure 1a). This effect typically reached a maximum around 1 mM Gd^{3+}, a concentration which increased basal NA secretion to 231 ($\pm$17)% of basal (mean$\pm$s.e.mean of effects from 20 cell preparations). With respect to evoked secretion, low concentrations of Gd^{3+}, from 1–100 μM, progressively inhibited 30 nM BK-induced secretion to near basal levels around 30 μM Gd^{3+} (Figure 1a). This inhibition was anticipated since Gd^{3+} is known to block many types of Ca^{2+}-permeant channel, including SOCs and VOCCs, and BK-induced secretion is dependent upon extracellular Ca^{2+} entry (see above). Unexpectedly, however, higher concentrations of 0.3–3 mM Gd^{3+} enhanced 30 nM BK-induced secretion to above control levels (Figure 1a). The effects of 1 and 3 mM Gd^{3+} and 30 nM BK were more than additive (see Figures 1a and 3a,b). This was in a [Gd^{3+}] range where all Ca^{2+} entry channels were expected to be blocked and no secretion above control was anticipated.

In contrast to the enhancement of BK-induced secretion, Gd^{3+} produced a simple inhibitory effect when catecholamine

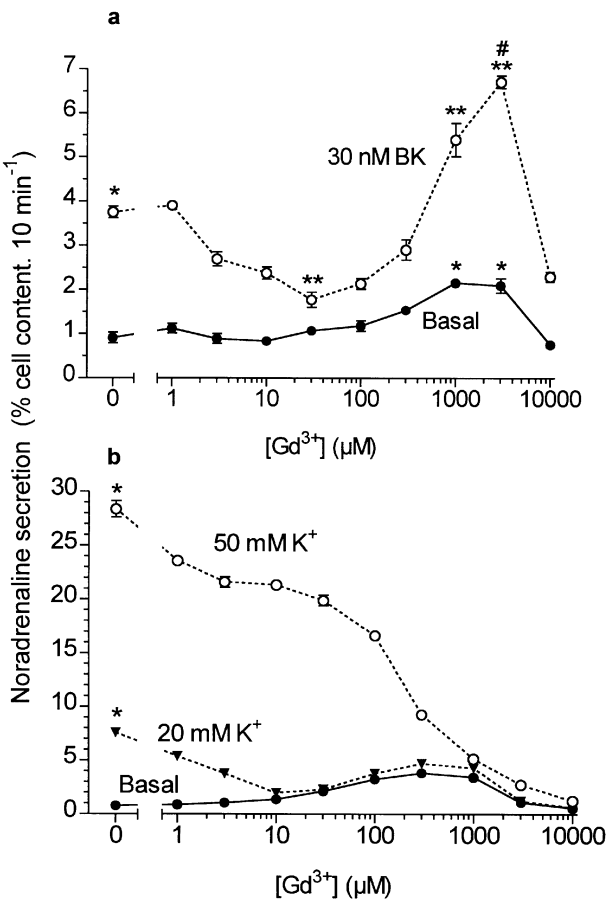

Figure 1 Effect of Gd^{3+} on noradrenaline secretion from bovine adrenal chromaffin cells in the absence and presence of (a) 30 nM bradykinin (BK), or (b) 20 or 50 mM K^+. Results are mean$\pm$s.e.mean for $n=4-6$ from a single cell preparation for each panel and are representative of similar results from three cell preparations. *$P<0.001$ compared with Basal secretion in the absence of Gd^{3+}. **$P<0.001$ compared with BK alone. #$P<0.01$ more than additive effect of Gd^{3+} and BK (Tukey-Kramer test; see Methods for test for interaction). Only selected comparisons are shown, for clarity.

secretion was evoked with the depolarizing stimulus, K^+. The response to 50 mM K^+ was reduced to basal levels in a concentration dependent manner by Gd^{3+} over the range of 1–1000 μM with an IC_{50} of around 100 μM (Figure 1b). Gd^{3+} was much more effective at inhibiting lower K^+ concentrations with an IC_{50} close to 3 μM against 20 mM K^+ and complete inhibition seen at 10–30 μM Gd^{3+} (Figure 1b).

Comparison of Gd^{3+} with the effects of other divalent and trivalent cations

In order to investigate further the enhancement of BK-induced catecholamine secretion by high Gd^{3+} concentrations, the effects of Gd^{3+} were compared with those of a variety of other tri- and di-valent metal ions. Two other lanthanides, Eu^{3+} and La^{3+}, displayed similar properties to Gd^{3+}. They produced the same enhancement of basal NA secretion as seen with Gd^{3+} (Figure 2a,b). Furthermore, low concentrations of Eu^{3+} or La^{3+} reduced 30 nM BK-induced secretion while mM concentrations enhanced 30 nM BK-induced secretion.

The pattern of effects of Gd^{3+}, Eu^{3+} and La^{3+} were not mimicked by the divalent cations Cd^{2+}, Co^{2+}, Ni^{2+} or Sr^{2+} (Figure 2c–f). Cd^{2+}, Co^{2+} and Ni^{2+} each inhibited BK-induced secretion with different potencies, while Sr^{2+} up to 3 mM had no effect. The inhibitory effect of Cd^{2+} on BK-induced secretion (Figure 2c) was similar to that reported previously (Owen *et al*., 1989b). The effects of these divalent cations were not investigated further here.

Role of extracellular and intracellular Ca^{2+} in the effects of Gd^{3+} on BK-induced catecholamine secretion

BK-induced secretion was dependent on extracellular Ca^{2+}, yet the enhancement of BK-induced secretion by Gd^{3+} was

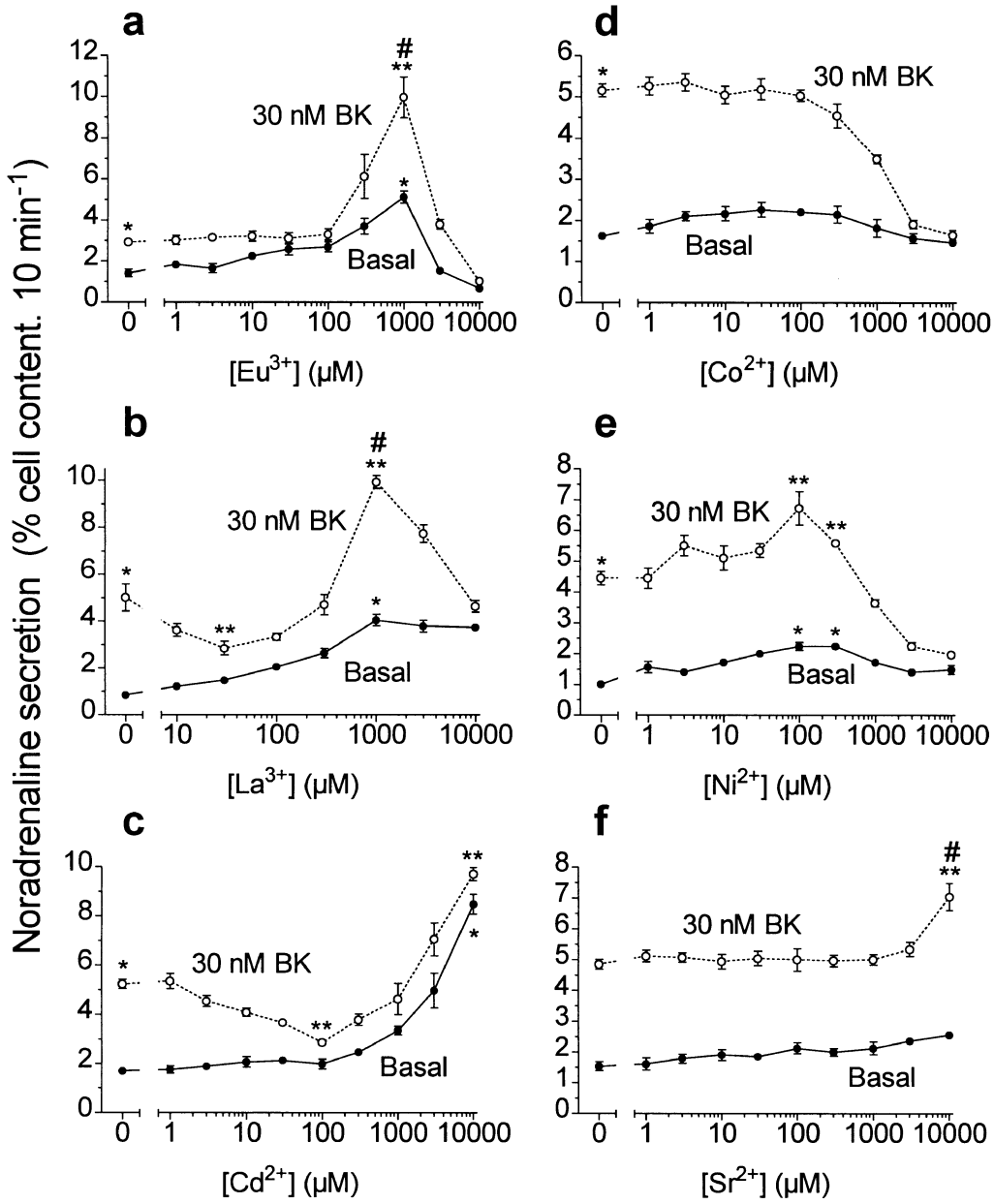

Figure 2 Effects of (a) Eu^{3+}, (b) La^{3+}, (c) Cd^{2+}, (d) Co^{2+}, (e) Ni^{2+} and (f) Sr^{2+} on noradrenaline secretion in the absence and presence of 30 nM bradykinin (BK). Results are mean ± s.e.mean for $n = 4–5$ from a single cell preparation for each panel and are representative of three cell preparations for each metal ion. $^*P < 0.001$ compared with Basal without any heavy metal ion, $^{**}P < 0.001$ compared with BK alone, $\#P < 0.01$ more than additive effect of BK and the heavy metal ion (Tukey-Kramer test; see Methods for test for interaction). Only selected comparisons are shown, for clarity.

seen at Gd^{3+} concentrations that were anticipated to block SOCs and VOCCs (Bourne & Trifaro, 1982; Canzoniero *et al*., 1993; Fernando & Barritt, 1994; 1995; Zimmermann, 1998). It was therefore of interest to determine whether Ca^{2+} was required for this effect. Surprisingly, Gd^{3+} also enhanced the ability of BK to induce catecholamine secretion in the absence of extracellular Ca^{2+} (Figure 3a). Under Ca^{2+}-free conditions, BK had little or no effect on noradrenaline secretion on its own, but in the presence of 1 mM Gd^{3+}, BK produced a substantial and robust secretory response despite the absence of Ca^{2+}. Gd^{3+} was also still able to increase basal catecholamine secretion in Ca^{2+}-free buffer (Figure 3a; see also Figure 4d).

One possible explanation for the effects of Gd^{3+} being independent of Ca^{2+} is that BK might open a Ca^{2+}-permeant channel in the membrane through which Gd^{3+} could enter the cell and itself evoke secretion. This would be consistent with the effects of another lanthanide, La^{3+}, which stimulates catecholamine secretion in permeabilized bovine chromaffin cells in the absence of Ca^{2+} (Powis *et al*., 1994). To ensure Gd^{3+} was not gaining access to the cytosol, the non-selective cation channel blocker SKF96365 was used. At 30 μM SKF96365 completely inhibited BK-induced catecholamine secretion (Figure 3b), suggesting it blocked all Ca^{2+} permeant channels opened by BK. However, in the presence of 30 μM SKF96365, Gd^{3+} was still able to enhance basal secretion and enhance the secretory effect of 30 nM BK (Figure 3b).

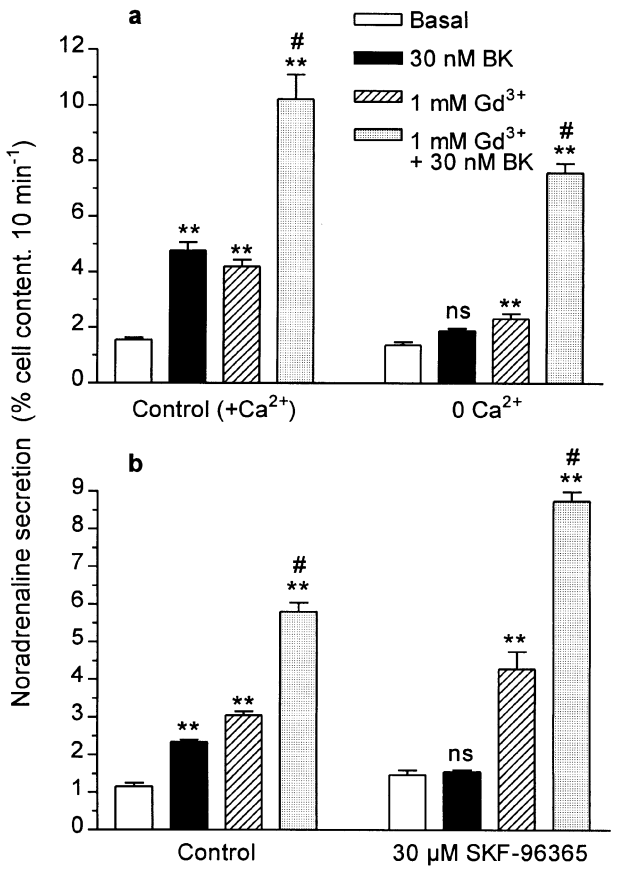

Figure 3 The effect of (a) Ca^{2+}-free buffer ($0Ca^{2+}$) and (b) 30 μM SKF96365, a non-selective cation channel inhibitor, on noradrenaline secretion induced by 30 nM bradykinin (BK) in the absence and presence of 1 mM Gd^{3+}. Results are mean$\pm$s.e.mean for $n=6-12$ from a single cell preparation for each panel and are representative of similar results from (a) four or (b) three cell preparations. ns $P>0.05$, $^{**}P<0.001$ compared with corresponding Basal, $\#P<0.05$ more than additive effects of BK and Gd^{3+} (Tukey-Kramer test; see Methods for test for interaction).

These results suggest that Gd^{3+} acts from the outside of the cell to enhance the secretory actions of BK. Since BK not only promotes extracellular Ca^{2+} influx but also mobilizes intracellular Ca^{2+} stores, the possible involvement of intracellular Ca^{2+} stores in the effects of Gd^{3+} was investigated. Gd^{3+} produced the same pattern of effects when secretion was evoked by other Ins(1,4,5)P_3-generating agonists, histamine and angiotensin II, with low Gd^{3+} concentrations inhibiting secretion and mM concentrations enhancing it (Figure 4a,b). Since these agonists, like BK, generate not only Ins(1,4,5)P_3 but also other second messengers that influence the secretory response, the effects of Gd^{3+} were also tested with caffeine, which mobilizes intracellular Ca^{2+} by a direct action on ryanodine receptors. Caffeine was very weak at evoking secretion on its own and sometimes failed to evoke any secretion (compare Figure 4c and d). However, in the presence of 1 mM Gd^{3+}, caffeine produced a substantial increase in secretion (Figure 4c). As with BK, this effect of Gd^{3+} on caffeine-induced secretion did not require extracellular Ca^{2+} (Figure 4d).

As shown above, the enhancing effect of Gd^{3+} did not depend on external Ca^{2+} entry and was seen with a variety of agents that mobilize internal Ca^{2+}. Therefore, the effect of depleting internal stores of Ca^{2+} was studied. Stores were depleted by pretreating cells in Ca^{2+}-free-buffer for 30 min either with 10 mM caffeine and 10 μM ryanodine, or with 100 nM thapsigargin. Gd^{3+} was then added for 15 min before the effect of BK was tested, still in the presence of store-depleting drugs in Ca^{2+}-free buffer. With this protocol, after 45 min in Ca^{2+} free buffer alone, 1 mM Gd^{3+} was unable to enhance basal secretion (Figure 5a,b; control panels) and was also unable to enhance 30 nM BK-induced secretion (data not shown). However it was able to enhance the stronger stimulus of 1 μM BK (Figure 5a,b: control panels). Following store depletion with caffeine and ryanodine or with thapsigargin, Gd^{3+} was no longer able to support catecholamine secretion by BK (Figure 5a,b).

Effect of Gd^{3+} on basal $^{45}Ca^{2+}$ efflux

The above results suggest that Gd^{3+} acts from the outside of the cell to enhance the effect of Ca^{2+} released from intracellular stores. A possible mechanism for this is through Gd^{3+} raising cytosolic Ca^{2+} levels by inhibiting the extrusion of Ca^{2+} from the cell. To test this possibility, $^{45}Ca^{2+}$ efflux was studied using chromaffin cells loaded to equilibrium with $^{45}Ca^{2+}$ (see Methods). The control $^{45}Ca^{2+}$ efflux rate did not change over the 12 min of collection, however the rate of $^{45}Ca^{2+}$ efflux was substantially reduced by 1 mM Gd^{3+} (Figure 6). Within 4 min of Gd^{3+} addition, this inhibition reached a steady state that averaged 53–62% inhibition over the final 4 min of Gd^{3+} exposure (range of effects from three cell preparations).

Measurement of cytosolic [Ca^{2+}] with Fura-2

To assess the effects of Gd^{3+} on cytosolic [Ca^{2+}], the fluorescence of Fura-2 in cells pre-loaded with Fura-2-acetoxymethyl ester was measured. During perfusion with Ca^{2+}-free buffer, 100 nM BK caused a transient rise in cytosolic [Ca^{2+}] that returned rapidly to resting levels within 2 min (Figure 7a). This response was abolished by prior depletion of the stores with thapsigargin (Figure 7d), confirming that it was due to mobilization of Ca^{2+} from intracellular stores. In the presence of Gd^{3+} (1 mM), the BK-induced increase in Fura-2 fluorescence was substantially

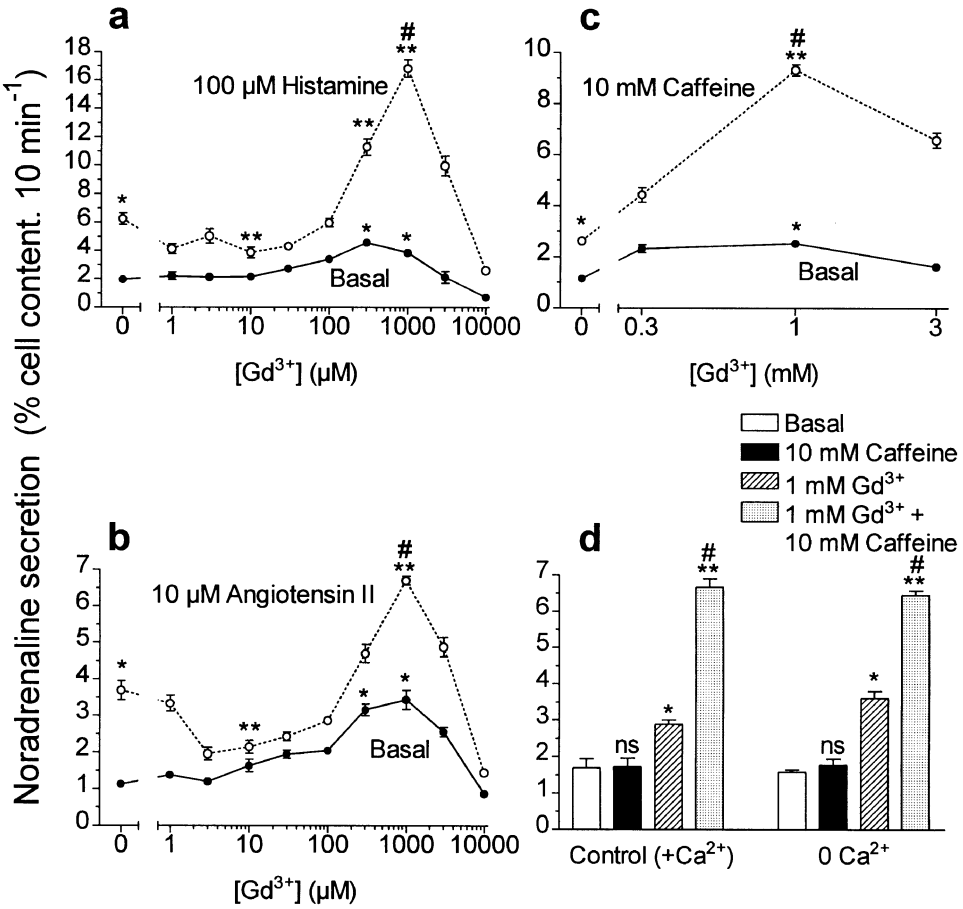

Figure 4 Effect of Gd^{3+} on noradrenaline secretion evoked by (a) 100 μM histamine, (b) 10 μM angiotensin II, and (c,d) 10 mM caffeine. (d) The dependence upon extracellular Ca^{2+} of the effect of Gd^{3+} on caffeine-induced secretion. Results are mean$\pm$s.e.mean for $n=4-5$ from a single cell preparation for each panel and are representative of similar data from three cell preparations for each treatment. ns $P>0.05$, $*P<0.001$ compared with corresponding Basal without Gd^{3+}, $**P<0.001$ compared with the response to histamine, angiotensin II or caffeine alone without Gd^{3+}, and $\#P<0.05$ more than additive effect of Gd^{3+} and the relevant agonist (Tukey-Kramer test: see Methods for test for interaction). Only selected comparisons are shown, for clarity.

prolonged, with the return to resting levels taking 10 min or longer and having a much longer time constant (Figure 7b). Gd^{3+} also increased the peak change in Fura-2 fluorescence evoked by BK by 27.7 ($\pm$8.8)% ($n=6$ experiments on three cell preparations: Figure 7c), however this failed to reach statistical significance ($P=0.056$, Student's t-test). Gd^{3+} on its own had little effect on resting Fura-2 fluorescence (Figure 7b). After store depletion with thapsigargin, BK was unable to increase Fura-2 fluorescence in the presence of Gd^{3+} (Figure 7e).

Discussion

The present data show that at low concentrations, Gd^{3+} can inhibit Ca^{2+}-dependent catecholamine secretion induced by BK from bovine chromaffin cells. By contrast, at concentrations from 300 μM – 3 mM, Gd^{3+} unexpectedly enhances the secretory response to BK. This latter effect does not require extracellular Ca^{2+}, is seen with a variety of agonists that mobilize Ca^{2+} from intracellular stores, and is abolished if these intracellular stores are depleted. Furthermore, Gd^{3+} inhibits Ca^{2+} efflux from these cells, and prolongs the cytosolic [Ca^{2+}] response to BK. Taken together, the results suggest that Gd^{3+} greatly enhances the normally very weak ability of Ca^{2+} mobilized from intracellular stores to evoke catecholamine secretion, by inhibiting its efflux from the cell. This indicates that intracellular Ca^{2+} stores in chromaffin cells are fully competent to support secretion at levels comparable to those supported by extracellular Ca^{2+} influx. The mechanism by which Ca^{2+} released from internal stores can evoke exocytosis is unknown and drugs such as the lanthanide ions that inhibit Ca^{2+} extrusion processes may be of value in its elucidation. The results also raise the possibility that cells may be able to powerfully modulate the efficacy with which intracellular Ca^{2+} stores can evoke secretion by regulating the plasma membrane mechanisms responsible for Ca^{2+} efflux.

Inhibitory effects of Gd^{3+} on catecholamine secretion

Low concentrations of Gd^{3+} reduced BK-induced secretion. Although secretion induced by BK is largely or fully dependent on extracellular Ca^{2+} (Kim & Westhead, 1989; O'Sullivan & Burgoyne, 1989; Owen *et al*., 1989b; present study), the nature of the channels that gate BK-induced Ca^{2+} influx are not known. BK depolarizes PC12 cells and chromaffin cells (Douglas *et al*., 1967; Fasolato *et al*., 1990). However BK-induced secretion is unaffected by inhibitors of L- and N-type VOCCs (Owen *et al*., 1989a,b; McMillian *et al*., 1992), but SOCs or other classes of VOCCs may be involved. The inhibition of BK-induced secretion by low Gd^{3+} concentrations is consistent with the involvement of such channels, since Gd^{3+} blocks these classes of channel (Demaurex *et al*., 1992; Fatatis *et al*., 1992; Canzaniero *et al*., 1993; Fernando &

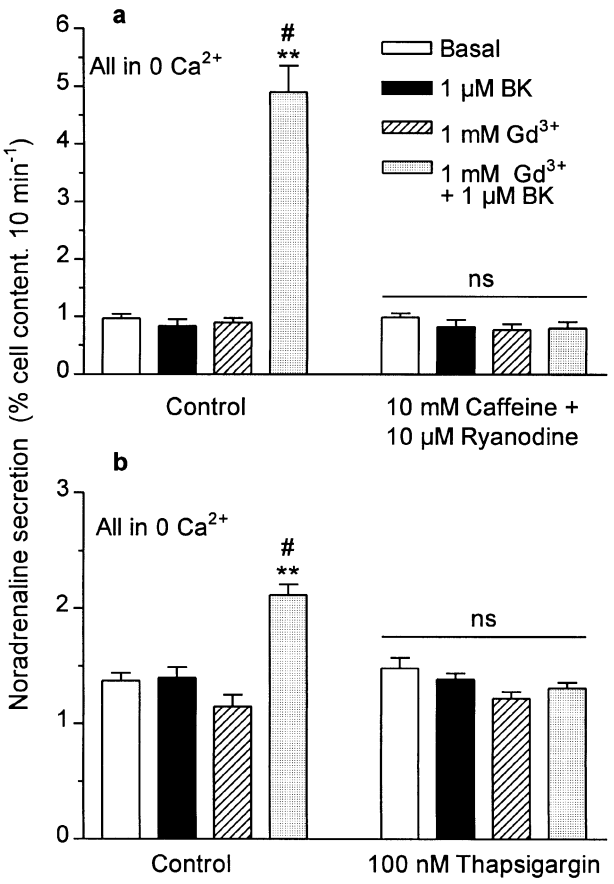

Figure 5 Effects of 1 mM Gd^{3+} and 1 μM bradykinin on noradrenaline secretion in Ca^{2+}-free buffer after depleting intracellular Ca^{2+} stores. Stores were depleted by pre-treating in Ca^{2+}-free buffer with (a) 10 mM caffeine and 10 μM ryanodine, or (b) 100 nM thapsigargin. All cells had been exposed to Ca^{2+}-free HBS for a total of 45 min before the 10 min stimulation period, in which secretion was measured (see Methods). Results are mean$\pm$s.e.mean for $n=5-6$ from a single cell preparation for each panel and are representative of similar results from three cell preparations for each condition. ns $P>0.05$ ANOVA, $^{**}P<0.001$ compared with corresponding Basal, $\#P<0.01$ more than additive effect of Gd^{3+} and BK (Tukey-Kramer test; see Methods for test for interaction). Only selected comparisons are shown, for clarity.

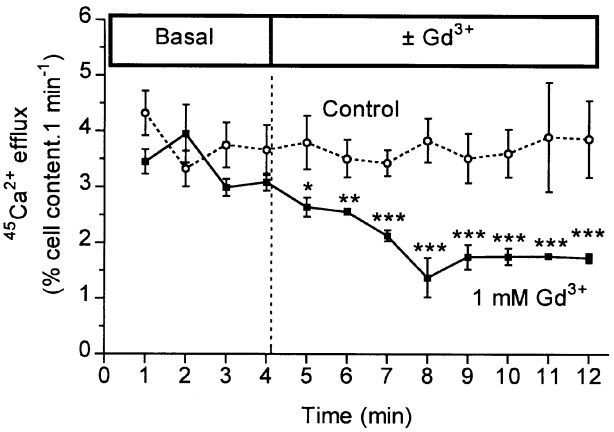

Figure 6 Efflux of $^{45}Ca^{2+}$ in the absence and presence of 1 mM Gd^{3+}. Cells preloaded with $^{45}Ca^{2+}$ were given eight 1 min washes, following which $^{45}Ca^{2+}$ efflux was measured over the twelve 1 min periods shown. Following four 1 min Basal collection periods, cells were exposed to 1 mM Gd^{3+} or control buffer as indicated. $^{45}Ca^{2+}$ efflux rate in each 1 min fraction is expressed as % cell content at the start of that fraction. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with the corresponding Basal fraction (Student's unpaired t-test).

Barritt, 1994; 1995; Zimmermann, 1998), but does not provide any further information on their identity. Their nature was not investigated further here.

Enhancement of catecholamine secretion by Gd^{3+}

At high concentrations, Gd^{3+} increased basal secretion and substantially potentiated catecholamine secretion induced by BK. Since BK-induced secretion is dependent on extracellular Ca^{2+} and these concentrations of Gd^{3+} are more than adequate to inhibit VOCCs (Figure 1b) and SOCs (see above), this enhancement was unexpected. The mechanism for this effect of Gd^{3+} is of significance for two reasons. First, in the presence of Gd^{3+} and absence of extracellular Ca^{2+}, BK was able to stimulate secretion to levels comparable to or greater than control responses to BK in the presence of Ca^{2+}. In chromaffin cells as in other excitable cells, substantial exocytosis is considered to require extracellular Ca^{2+} entry, at least in part through VOCCs. In the absence of extracellular Ca^{2+}, secretion is abolished or reduced to very low levels (see Burgoyne, 1991). The present results show that in the presence of Gd^{3+}, mobilization of intracellular Ca^{2+} stores is itself fully capable of supporting secretion at levels comparable to those normally seen only when extracellular Ca^{2+} is available. Secondly, the effect of Gd^{3+} is seen with a range of agents that mobilize intracellular Ca^{2+}, including caffeine. This suggests that the effect of Gd^{3+} does not require stimulation of receptors or G proteins or the production of second messengers.

The enhancing effect of Gd^{3+} was mimicked by Eu^{3+} and La^{3+} but not by several divalent cations Sr^{2+}, Co^{2+} and Ni^{2+}. Together with the low potency of Gd^{3+}, this makes it unlikely the effect of Gd^{3+} is mediated by receptors for heavy metal ions, which have been described on many other cell types (see McNulty & Taylor, 1999). Several lanthanide ions were seen to have similar actions on histamine release from mast cells, which was inhibited by low concentrations and enhanced by high (Pearce & White, 1981). Two possible mechanisms might explain the enhancing effect of Gd^{3+}. First, BK might activate a membrane ion channel through which Gd^{3+} enters the cell and acts intracellularly to evoke secretion. Secondly, Gd^{3+} might act from outside the cell to enhance the intracellular actions of BK that cause secretion.

Since it mobilizes intracellular Ca^{2+}, it is likely BK activates SOCs in the chromaffin cell membrane (Powis *et al*., 1996). It is probable these are Ca^{2+}-permeant, non-selective cation channels (Parekh & Penner, 1997; Barritt, 1999). Gd^{3+} might be able to pass through such channels and, once inside, substitute for Ca^{2+} to elicit secretion (as can La^{3+}: Powis *et al*., 1994; Tomsig & Suszkiw, 1996). This explanation, however, is not consistent with the data obtained with SKF96365. This is an unselective inhibitor of cation channels and is known to inhibit VOCCs and SOCs and Ca^{2+}-permeant receptor-operated channels such as nicotinic receptor channels (see Tachikawa *et al*., 1994). SKF96365 completely blocked the Ca^{2+}-dependent secretory response to BK, indicating it blocked all Ca^{2+}-permeant channels activated by BK. Yet SKF96365 failed to block the ability of Gd^{3+} to enhance basal and BK-induced secretion (Figure 3b). This does not support the suggestion that Gd^{3+} is gaining access to the cell and acting from inside.

The most probable explanation for the present results is that high concentrations of Gd^{3+} act from the outside of the cell to enhance the efficacy of Ca^{2+} released from intracellular stores in eliciting secretion. The key requirement for exocytosis appears to be a high local $[Ca^{2+}]$ in the subplasmalemmal

zone, rather than a particular temporal change in cytosolic [Ca^{2+}] (Burgoyne, 1991). It is therefore likely that the main action of Gd^{3+} is to cause accumulation of Ca^{2+} in this spatially restricted zone. The present results indicate that Gd^{3+} achieves this by inhibiting Ca^{2+} export from the cell, by inhibiting either the Na^{+}/Ca^{2+}-exchanger or the plasma membrane Ca^{2+}-ATPase or both. This is consistent with previous studies that showed the related lanthanide La^{3+} inhibits both Ca^{2+} export pathways in bovine chromaffin cells (Powis *et al*., 1994).

Gd^{3+} substantially prolonged the duration of elevated Ca^{2+} in response to BK (Figure 7). Although this effect is consistent with the ability of Gd^{3+} to reduce $^{45}Ca^{2+}$ efflux (Figure 6; see also Bourne & Trifaro, 1982), it is unlikely this causes the enhancement of exocytosis produced by Gd^{3+} (see above). However, no significant increase in peak [Ca^{2+}] was detected in

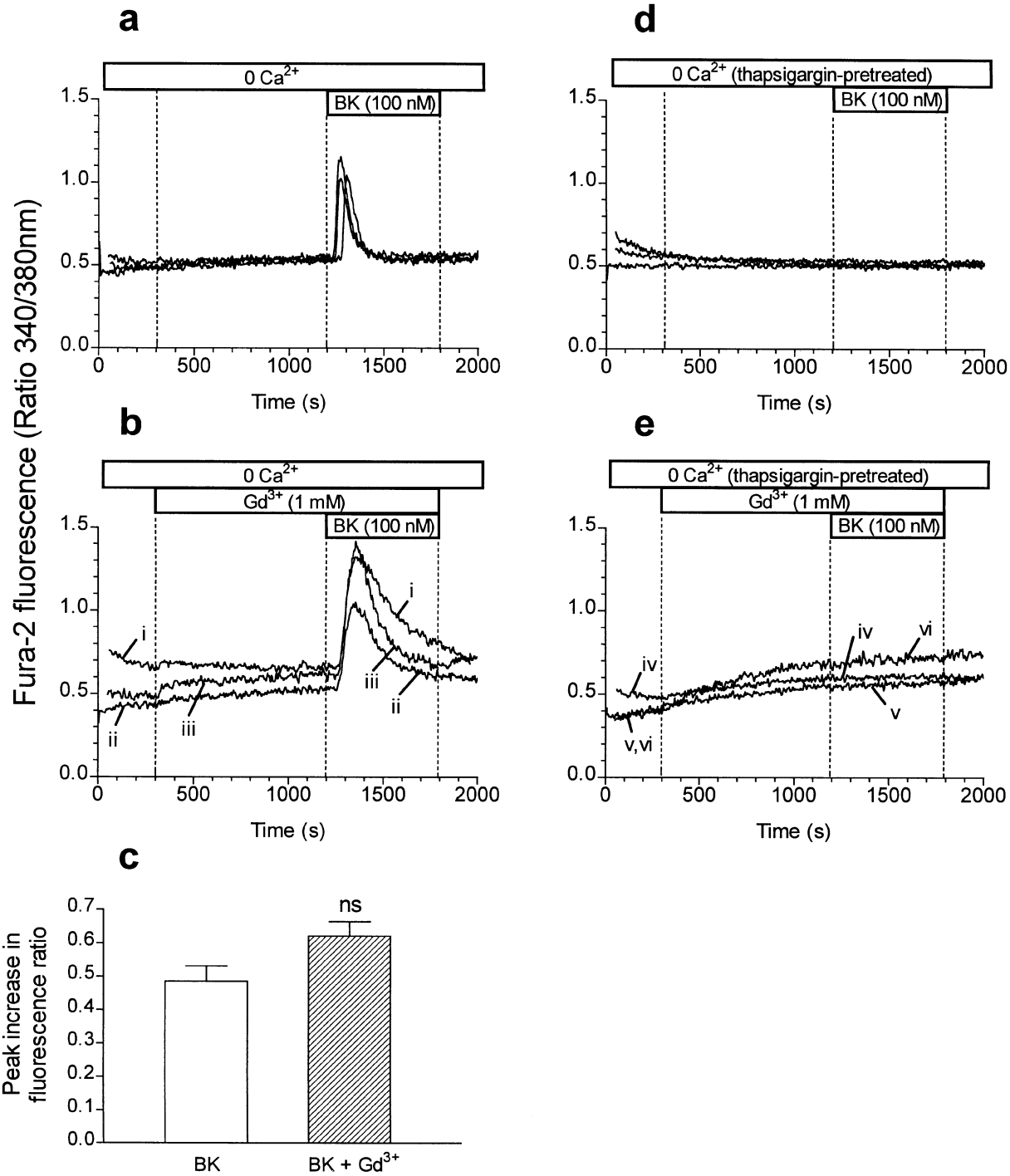

Figure 7 Effects of Gd^{3+} and prior store depletion with thapsigargin on bradykinin-induced changes in cytosolic [Ca^{2+}] measured with Fura-2 in Ca^{2+}-free buffer. Cells loaded with Fura-2-acetoxymethyl ester were pretreated for 30 min in Ca^{2+}-free buffer in the absence (a,b) or presence (d,e) of 100 nM thapsigargin. After recording resting Fura-2 fluorescence for 5 min, cells in (b) and (e) were exposed to 1 mM Gd^{3+} in the perfusion fluid for 15 min. Cells were then stimulated with 100 nM BK for 10 min. The ratio (340 nm/380 nm) of fluorescence emitted at 510 nm while exciting at 340 nm and 380 nm is presented, after correction for autofluorescence. The three traces in panels a, b, d and e are representative results from three different cell preparations: individual traces in (b) and (e) are labelled i–vi for clarity. Panel (c) shows the peak increase in Fura-2 fluorescence ratio evoked by 100 nM BK in the absence and presence of 1 mM Gd^{3+}. The data are mean (±s.e.mean) for six determinations from three cell preparations (ns, $P>0.05$ compared to BK alone, Student's *t*-test).

the presence of Gd^{3+} (Figure 7c). Furthermore, in the absence of BK, Gd^{3+} increased basal catecholamine secretion but had little effect on resting cytosolic $[Ca^{2+}]$ (Figure 7b). It is possible that Gd^{3+} has significant effects on the local peak $[Ca^{2+}]$ in the subplasmalemmal zone that are not reflected in the average Fura-2 fluorescence of populations of cells. Previous studies have shown that such localized increases in $[Ca^{2+}]$ are very poorly detected by fluorescent dye measurements (Tse *et al*., 1997). Under basal conditions, Gd^{3+} may enhance the size and duration of localized, transient increases in $[Ca^{2+}]$ due to the asynchronous, spontaneous release of Ca^{2+} from parts of the endoplasmic reticulum located close to the plasma membrane. Such a localized and transient alteration in cytosolic Ca^{2+} may not be reflected in the spatially and temporally averaged $[Ca^{2+}]$ detected by Fura-2. Similarly, when store Ca^{2+} is mobilized in the presence of Gd^{3+}, the failure to detect a significant enhancement in peak $[Ca^{2+}]$ is also likely to be due to Fura-2 spatially averaging the changes in $[Ca^{2+}]$ in the population of cells. A similar conclusion was reached in studies on pituitary gonadotrophs, in which store Ca^{2+} supported exocytosis by raising $[Ca^{2+}]$ to high levels near exocytotic sites, without these local high concentrations of Ca^{2+} being detected by Indo-1 or Fura-2 (Tse *et al*., 1997).

It is therefore likely that Gd^{3+} greatly enhances the efficacy of Ca^{2+} released by BK from intracellular stores in eliciting catecholamine secretion, by inhibiting Ca^{2+} export from the cytosol through the plasma membrane. A similar dramatic enhancement of the secretory responses to BK, histamine and other weak secretagogues that mobilize intracellular Ca^{2+} was seen when the Na^+/K^+-ATPase was inhibited with ouabain (see Houchi *et al*., 1997). However, unlike the effect of Gd^{3+}, this action of ouabain was dependent on extracellular Ca^{2+} and appeared to be due to intracellular accumulation of Na^+ to levels that reverse the Na^+/Ca^{2+} exchanger, so enhancing Ca^{2+} entry.

The present results indicate that the influence of Ca^{2+} export on the cytosolic $[Ca^{2+}]$ or on its distribution following store mobilization must be substantial, since mobilizing intracellular Ca^{2+} is normally a very weak or ineffective means of eliciting secretion (see Figures 3a and 4c,d). This suggests that, since cells regulate the activity of the Ca^{2+} export processes (Matsuda *et al*., 1997; Guerini, 1998), they may have the capacity to dramatically modulate the efficacy of intracellular Ca^{2+} stores in evoking exocytosis. Three isoforms of the Na^+/Ca^{2+}-exchanger have recently been cloned from bovine adrenal medulla (Pan *et al*., 1998). The present results suggest that, together with the plasma membrane Ca^{2+}-ATPases, these would be interesting targets for the development of specific pharmacological inhibitors. Such drugs would greatly assist investigation of the roles these export processes play in modulating Ca^{2+}-dependent responses and of the physiological mechanisms by which cells might regulate the different Ca^{2+} export pathways. Gd^{3+} has many actions in addition to inhibition of these Ca^{2+} efflux pathways (see Caldwell *et al*., 1998). However, in the absence of more selective drugs, it should prove useful in helping to elucidate the mechanism by which Ca^{2+} released from internal stores can elicit secretion (e.g., in the determination of changes in the spatial Ca^{2+} signals during store mobilization while Ca^{2+} efflux is inhibited).

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References

BALES, P.J.R. & MARLEY, P.D. (1998). Effect of gadolinium on bradykinin induced catecholamine secretion from bovine adrenal chromaffin cells. *Proc. Aust. Soc. Clin. Exp. Pharmacol. Toxicol.,* **5,** Oral 6 – 5.

BARRITT, G.J. (1999). Receptor-activated Ca^{2+} inflow in animal cells: a variety of pathways tailored to meet different intracellular Ca^{2+} signalling requirements. *Biochem. J.,* **337,** 153 – 169.

BOURNE, G.W. & TRIFARÓ, J.M. (1982). The gadolinium ion: A potent blocker of calcium channels and catecholamine release from cultured chromaffin cells. *Neuroscience,* **7,** 1615 – 1622.

BURGOYNE, R.D. (1991). Control of exocytosis in adrenal chromaffin cells. *Biochim. Biophys. Acta,* **1071,** 174 – 202.

CALDWELL, R.A., CLEMO, H.F. & BAUMGARTEN, C.M. (1998). Using gadolinium to identify stretch-activated channels: technical considerations. *Am. J. Physiol.,* **275,** C619 – C621.

CANZONIERO, L.M.T., TAGLIATELA, M., DIRENZO, G. & ANNUNZIATO, L. (1993). Gadolinium and neomycin block voltage-sensitive Ca^{2+} channels without interfering with the Na^+-Ca^{2+} antiporter in brain nerve endings. *Eur. J. Pharmacol.,* **245,** 97 – 103.

CASTRO, E., MATEO, J., TOME, A.R., BARBOSA, R.M., MIRAS-PORUGAL, M.T. & ROSARIO, L.M. (1995). Cell-specific purinergic receptors coupled to Ca^{2+} entry and Ca^{2+} release from internal stores in adrenal chromaffin cells. *J. Biol. Chem.,* **270,** 5098 – 5106.

CHALLISS, R.A.J., JONES, J.A., OWEN, P.J. & BOARDER, M.R. (1991). Changes in inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate mass accumulation in cultured adrenal chromaffin cells in response to bradykinin and histamine. *J. Neurochem.,* **56,** 1083 – 1086.

CHEEK, T.R. & BARRY, V.A. (1993). Stimulus-secretion coupling in excitable cells: a central role for calcium. *J. Exp. Biol.,* **184,** 183 – 196.

CHEEK, T.R. & THASTRUP, O. (1989). Internal Ca^{2+} mobilization and secretion in bovine adrenal chromaffin cells. *Cell Calcium,* **10,** 213 – 221.

D'ANDREA, P., ZACCHETTI, D., MELDOLESI, J. & GROHOVAZ, F. (1993). Mechanism of $[Ca^{2+}]_i$ oscillations in rat chromaffin cells. *J. Biol. Chem.,* **268,** 15213 – 15220.

DEMAUREX, N., LEW, D.P. & KRAUSE, K. (1992). Cyclopiazonic acid depletes intracellular Ca^{2+} stores and activates an influx pathway for divalent cations in HL-60 cells. *J. Biol. Chem.,* **267,** 2318 – 2324.

DOUGLAS, W.W., KANNO, T. & SAMPSON, S.R. (1967). Effects of acetylcholine and other medullary secretagogues and antagonists on the membrane potential of adrenal chromaffin cells: an analysis employing techniques of tissue culture. *J. Physiol.,* **188,** 107 – 120.

FASOLATO, C., PIZZO, P. & POZZAN, T. (1990). Receptor-mediated calcium influx in PC12 cells. *J. Biol. Chem.,* **265,** 20351 – 20355.

FATATIS, A., BASSI, A., MONSURRO, M.R., SORRENTINO, G., MITA, G.D., DIRENZO, G.F. & ANNUNZIATO, L. (1992). Lan-1: A human neuroblastoma cell line with M_1 and M_3 muscarinic receptor subtypes coupled to intracellular Ca^{2+} elevation and lacking Ca^{2+} channels activated by membrane depolarization. *J. Neurochem.,* **59,** 1 – 9.

FERNANDO, K.C. & BARRITT, G.J. (1994). Characterisation of the inhibition of the hepatocyte receptor-activated Ca^{2+} inflow system by gadolinium and SK&F 96365. *Biochim. Biophys. Acta,* **1222,** 383 – 389.

FERNANDO, K.C. & BARRITT, G.J. (1995). Characterisation of the divalent cation channels of the hepatocyte plasma membrane receptor-activated Ca^{2+} inflow system using lanthanide ions. *Biochim. Biophys. Acta.,* **1268,** 97 – 106.

FINNEGAN, J.M., BORGES, R. & WIGHTMAN, R.M. (1996). Comparison of cytosolic Ca^{2+} and exocytosis responses from single rat and bovine chromaffin cells. *Neuroscience,* **71,** 833–843.

FINNEGAN, J.M. & WIGHTMAN, R.M. (1995). Correlation of real-time catecholamine release and cytosolic Ca^{2+} at single bovine chromaffin cells. *J. Biol. Chem.,* **270,** 5353–5359.

GUERINI, D. (1998). The significance of the isoforms of plasma membrane calcium ATPase. *Cell Tiss. Res.,* **292,** 191–197.

HAYCOCK, J.W. (1993). Multiple signaling pathways in bovine chromaffin cells regulate tyrosine hydroxylase phosphorylation at Ser^{19}, Ser^{31}, and Ser^{40}. *Neurochem. Res.,* **18,** 15–26.

HOUCHI, H., MASUDA, Y., ISHIMURA, Y., OHUCHI, T., MURADUMO, Y. & OKA, M. (1994). Calcium efflux from cultured bovine adrenal chromaffin cells induced by bradykinin. *Biochem. Pharma.,* **47,** 1309–1313.

HOUCHI, H., YOSHIZUMI, M., MINAKUCHI, K., ISHIMURA, Y., MORITA, K., TAMAKI, T. & OKA, M. (1997). Potentiation of histamine-induced catecholamine secretion by ouabain in cultured bovine adrenal chromaffin cells is dependent on calcium and sodium influx. *Life Sci.,* **60,** 2051–2058.

KIM, K. & WESTHEAD, E.W. (1989). Cellular responses to Ca^{2+} from extracellular and intracellular sources are different as shown by simultaneous measurements of cytosolic Ca^{2+} and secretion from bovine chromaffin cell. *Cell Biol.,* **86,** 9881–9885.

KIM, S.J. & KIM, J. (1998). Relation of exocytosis and Ca^{2+}-activated K^+ current during Ca^{2+} release from intracellular stores in individual rat chromaffin cells. *Brain Res.,* **799,** 197–206.

LIU, P., LIN, Y.& KAO, L. (1991). Caffeine-sensitive calcium stores in bovine adrenal chromaffin cells. *J. Neurochem.,* **56,** 172–177.

LIVETT, B.J., MARLEY, P.D., MITCHELHILL, K.I., WAN, D.C.C. & WHITE, T.D. (1987a). Assessment of adrenal chromaffin cell secretion: presentation of four techniques. In: Poisner, A.M. & Trifaro, J.M. (eds). *In Vitro techniques for studying secretion.* CRC Press: Boca Raton, FL, pp. 177–204.

LIVETT, B.J., MITCHELHILL, K.I. & DEAN, D.M. (1987b). Adrenal chromaffin cells: their isolation and culture. In: Poisner, A.M. & Trifaro, J.M. (eds). *In Vitro techniques for studying secretion.* CRC Press: Boca Raton, FL, pp. 171–176.

MARLEY, P.D., BALES, P.J.R., O'FARRELL, M., POWIS, D.A. & ZERBES, M. (1999). La^{3+} and Gd^{3+} enhance the efficacy of intracellular Ca^{2+} release in evoking catecholamine secretion from bovine chromaffin cells. *Proc. Aust. Neurosci. Soc.,* **10,** Oral 15–4.

MATSUDA, T., TAKUMA, K. & BABA, A. (1997). Na^+-Ca^{2+} exchanger: physiology and pharmacology. *Jpn. J. Pharmacol.,* **74,** 1–20.

MCMILLIAN, M.K., TUOMINEN, R.K., HUDSON, P.M., SUH, H.H. & HONG, J.S. (1992). Angiotensin II receptors are coupled to ω-conotoxin-sensitive calcium influx in bovine adrenal medullary chromaffin cells. *J. Neurochem.,* **58,** 1285–1291.

MCNULTY, T.J. & TAYLOR, C.W. (1999). Extracellular heavy-metal ions stimulate Ca^{2+} mobilization in hepatocytes. *Biochem. J.,* **339,** 555–561.

MOELLER, I., BUNN, S.J. & MARLEY, P.D. (1989). Actions of somatostatin on perfused bovine adrenal glands and cultured bovine adrenal medullary cells. *Brain Res.,* **484,** 192–202.

O'SULLIVAN, A.J. & BURGOYNE, R.D. (1989). A comparison of bradykinin, angiotensin II and muscarine in stimulation of cultured bovine adrenal chromaffin cells. *Biosci. Rep.,* **9,** 243–252.

OWEN, P.J., MARRIOTT, D.B. & BOARDER, M.R. (1989a). Evidence for a dihydropyridine-sensitive and conotoxin-insensitive release of noradrenaline and uptake of calcium in adrenal chromaffin cells. *Br. J. Pharmacol.,* **97,** 133–138.

OWEN, P.J., PLEVIN, R. & BOARDER, M.R. (1989b). Characterization of bradykinin-stimulated release of noradrenaline from cultured bovine adrenal chromaffin cells. *J. Pharmacol. Exp. Ther.,* **248,** 1231–1236.

PAN, C., CHU, Y. & KAO, L. (1998). Molecular study of the Na^+/Ca^{2+} exchanger in bovine adrenal chromaffin cells. *Biochem. J.,* **336,** 305–310.

PAREKH, A.B. & PENNER, R. (1997). Store depletion and calcium influx. *Physiol. Rev.,* **77,** 901–930.

PEARCE, F.L. & WHITE, J.R. (1981). Effect of lanthanide ions on histamine secretion from rat peritoneal mast cells. *Br. J. Pharmacol.,* **72,** 341–347.

PLEVIN, R. & BOARDER, M.R. (1988). Stimulation of formation of inositol phosphates in primary cultures of bovine adrenal chromaffin cells by angiotensin II, histamine, bradykinin and carbochol. *J. Neurochem.,* **51,** 634–641.

POWIS, D.A., CLARK, C.L. & O'BRIEN, K.J. (1994). Lanthanum can be transported by the sodium-calcium exchange pathway and directly triggers catecholamine release from bovine chromaffin cells. *Cell Calcium,* **16,** 377–390.

POWIS, D.A., CLARK, C.L. & O'BRIEN, K.J. (1996). Depleted internal store-activated Ca^{2+} entry can trigger neurotransmitter release in bovine chromaffin cells. *Neurosci. Lett.,* **204,** 165–168.

ROBINSON, I.A. & BURGOYNE, R.D. (1991). Characterization of distinct inositol 1,4,5-trisphosphate-sensitive and caffeine-sensitive calcium stores in digitonin-permeabilized adrenal chromaffin cells. *J. Neurochem.,* **56,** 1587–1593.

ROBINSON, I.A., CHEEK, T.R. & BURGOYNE, R.D. (1992). Ca^{2+} influx induced by the Ca^{2+}-ATPase inhibitors 2,5-di-(*t*-butyl)-1,4-benzohydroquinone and thapsigargin in bovine adrenal chromaffin cells. *Biochem. J.,* **288,** 457–463.

TACHIKAWA, E., TAKAHASHI, S., MIZUMA, K., KONDO, Y., KASHIMOTO, T. & TAKAHASHI, E. (1994). Effects of SK&F 96365, an inhibitor of receptor-mediated Ca^{2+} entry, on Ca^{2+} influx and catecholamine secretion in bovine adrenal chromaffin cells. *Neurosci. Lett.,* **177,** 155–158.

TANAKA, K., SHIBUYA, I., UEZONO, Y., UETA, Y., TOYOHIRA, Y., YANAGIHARA, N., IZUMI, F., KANNO, T. & YAMASHITA, H. (1998). Pituitary adenylate cyclase-activating polypeptide causes Ca^{2+} release from ryanodine/caffeine stores through a novel pathway independent of both inositol trisphosphates and cyclic AMP in bovine adrenal medullary cells. *J. Neurochem.,* **70,** 1652–1661.

TOMSIG, J.L. & SUSZKIW, J.B. (1996). Metal selectivity of exocytosis in α-toxin-permeabilized bovine chromaffin cells. *J. Neurochem.,* **66,** 644–650.

TSE, F.W., TSE, A., HILLE, B., HORSTMANN, H. & ALMERS, W. (1997). Local Ca^{2+} release from internal stores controls exocytosis in pituitary gonadotrophs. *Neuron,* **18,** 121–132.

ZERBES, M., BUNN, S.J. & POWIS, D.A. (1998). Histamine causes Ca^{2+} entry via both a store-operated and a store-independent pathway in bovine adrenal chromaffin cells. *Cell Calcium,* **23,** 379–386.

ZIMMERMANN, B. (1998). Calcium store depletion activates two distinct calcium entry pathways in secretory cells of the blowfly salivary gland. *Cell Calcium,* **23,** 53–63.

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