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Ca^{2+} influx stimulated phospholipase C activity in bovine adrenal chromaffin cells: responses to K^+ depolarization and histamine

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Abstract

The role of Ca^{2+} influx in activating phospholipase C in bovine adrenal chromaffin cells has been investigated. Phospholipase C activity in response to K^+ depolarization (56 mM) was blocked by the L-type Ca^{2+} channel antagonist nifedipine and partially inhibited by the ω-conotoxins GVIA and MVIIC. In contrast, phospholipase C activity in response to histamine receptor activation was unaffected by ω-conotoxin GVIA and partially inhibited by ω-conotoxin MVIIC or nifedipine. This response was however markedly inhibited by the non-selective Ca^{2+} channel antagonists La^{3+} or 1-[β-[3-(4-Methoxyphenyl)propoxy]-4-methoyphenethyl]-*H*-imidazole (SKF-96365). Despite this Ca^{2+} dependence phospholipase C activity was not increased during periods of ''capacitative'' Ca^{2+} inflow generated by histamine-, caffeine- or thapsigargin-mediated depletion of internal Ca^{2+} stores. Thus, while Ca^{2+} influx in response to K^+ depolarization or G-protein receptor activation can increase phospholipase C activity in these cells, in the latter case it appears to be ineffective unless there is concurrent agonist occupation of the receptor.

Keywords: Phospholipase C; Histamine; Chromaffin cell; Ca^{2+}; Inositol phospholipid; Adrenal medulla

1. Introduction

Stimulation of certain G-protein coupled receptors (histamine H_1, muscarinic, angiotensin II and bradykinin) on bovine adrenal medullary chromaffin cells increases the activity of inositol phospholipid-specific phospholipase C (Plevin and Boarder, 1988; Sasakawa et al., 1989; Bunn et al., 1990). In addition, previous data has demonstrated that Ca^{2+} influx following nicotinic or high K^+ mediated depolarization, or the direct elevation of Ca^{2+} in permeabilized chromaffin cells, also results in increased activity of this enzyme (Eberhard and Holz, 1988; Sasakawa et al., 1989). Activated phospholipase C then mediates the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate, generating the intracellular signalling molecules *sn*-1,2-diacylglycerol and inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$). Diacylglycerol is known to activate certain protein kinase C isozymes while $Ins(1,4,5)P_3$ mobilizes Ca^{2+} from intracellular stores (Lee and Rhee, 1995; Singer et al., 1997).

The addition of exogenous $Ins(1,4,5)P_3$ to permeabilized chromaffin cells causes the release of Ca^{2+} from intracellular stores. Similarly, activation of phospholipase C-coupled receptors on these cells elevates the intracellular free Ca^{2+} concentration even in the absence of extracellular Ca^{2+} (Stauderman and Pruss, 1990; Robinson and Burgoyne, 1991; Zerbes et al., 1998). While the role of this intracellular Ca^{2+} signal in chromaffin cells is not yet fully established we and others have shown that mobilization of Ca^{2+} in response to histamine appears able to support a brief period of catecholamine secretion and the site-specific phosphorylation of tyrosine hydroxylase (Bunn and Boyd, 1992; Bunn et al., 1995a; Zhang et al., 1995). It is also probable that depletion of intracellular Ca^{2+} stores by histamine and other phospholipase C-coupled receptors triggers a secondary, or so-called capacitative, Ca^{2+} influx across the plasma membrane (Powis et al., 1996; Zerbes et al., 1998; Barritt, 1999; Putney and McKay, 1999).

Histamine is a potentially significant regulator of adrenal chromaffin cell function. The cells of the bovine adrenal medulla express both H_1 and H_2 receptors although only

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the former appear to be coupled to phospholipase C activity and catecholamine release (Livett and Marley, 1986; Plevin and Boarder, 1988; Bunn and Boyd, 1992). The mechanism by which histamine evokes catecholamine secretion is unknown although it is widely assumed that activation of phospholipase C is in some way involved in the initiation of this response. It is well established that the maintenance of both histamine-induced secretion and phospholipase C activation beyond the first few seconds of stimulation requires the influx of extracellular Ca^{2+} (Bunn and Boyd, 1992; Bunn et al., 1995b). Previous studies have investigated the nature of the Ca^{2+} channels, which support histamine-induced secretion, and in a number of cases have compared them with the Ca^{2+} channels involved with secretion induced by K^+ depolarization (Noble et al., 1988a; Goh and Kurosawa, 1991; Cheek et al., 1993, 1994; Firestone and Browning, 1994). There is however little information available regarding the nature of the Ca^{2+} channels which support phospholipase C activation to either of these stimuli. This is a potentially important issue because recent evidence has indicated that while histamine-induced catecholamine secretion and histamine-induced activation of the catecholamine synthesising enzyme tyrosine hydroxylase in these cells are both dependent upon Ca^{2+} influx, the two responses may involve different populations of pharmacologically distinct Ca^{2+} channels (O'Farrell and Marley, 1999). The current study extends our initial observations with regard to the Ca^{2+} dependence of the histamine mediated phospholipase C response and provides a partial characterization of the Ca^{2+} channels involved with the histamine response in comparison to those activated by depolarization with high K^+. A preliminary report of some of this work has been published previously (Bunn and Dunkley, 1997).

2. Materials and methods

2.1. Isolation and culture of bovine adrenal medullary chromaffin cells

Adrenal medullary chromaffin cells were isolated from bovine adrenal glands by protease/collagenase digestion as described previously (Zerbes et al., 1998). The isolated cell suspension was first purified by centrifugation through a cushion of 4% (w/v) bovine serum albumin in Locke's buffer ($100 \times g$ for 10 min) and then subjected to the differential plating procedure described by Owen et al., 1989. The resulting purified adrenal chromaffin cell suspension was plated onto rat tail collagen-coated 24-well tissue culture plates at a density of 0.5×10^6 cells per well, in Dulbecco's modified Eagles medium (DMEM) supplemented with 15 mM HEPES, 4 mM L-glutamine, 5 mM glucose, 10% v/v fetal calf serum, 25 μg/ml each of fluorodeoxyuridine, cytosine arabinoside, 5 μg/ml nystatin, 50 μg/ml gentamycin and 100 μg/ml each of penicillin G and streptomycin (pH 7.2). Cells required for fura-2 fluorimetry were prepared as above but then plated onto six-well tissue culture plates containing two flamed glass coverslips (9×22 mm) at a density of 10^6 cells per well. The cell cultures were maintained at 37°C in an incubator containing 5% CO_2 in humidified air. After 3 days in culture and every third day thereafter the cells were fed by replacing the culture medium with 1 ml of fresh DMEM (prepared as above). Cells were used after between 3 and 12 days in culture.

2.2. Stimulation and measurement of phospholipase C activity

For the measurement of phospholipase C activity the cells were first loaded for 48 h with [^{3}H]inositol. The DMEM culture medium was removed and replaced with 500 μl of medium M199 supplemented with 26 mM $NaHCO_3$, 15 mM HEPES, 2% v/v fetal calf serum and antibiotics as described above, containing 5 μCi/ml of [^{3}H]inositol (pH 7.2). After [^{3}H]inositol loading the cells were washed twice (5 min each wash) with 1 ml buffer composed of 149 mM NaCl, 2.6 mM KCl, 2.15 mM K_2HPO_4, 0.85 mM KH_2PO_4, 1.18 mM $MgSO_4$, 2.2 mM $CaCl_2$, 5 mM LiCl, 10 mM glucose and 0.1% w/v bovine serum albumin at pH 7.4 and 37°C. The second 5-min wash was removed and replaced with 1 ml of a histamine, or high K^+, stimulating solution (wash buffer containing 10 μM histamine or 56 mM K^+ with a corresponding reduction in NaCl concentration). In experiments examining the effect of Ca^{2+} channel antagonists a 10-min preincubation period in the presence of these agents was introduced between the second wash and the stimulation period. After the required stimulation time (routinely 10 min) the incubation was terminated by replacing the buffer with 500 μl 10% (w/v) perchloric acid.

For experiments with permeabilized cells the cultures were first washed twice (5 min each wash) with 1 ml of Ca^{2+} free buffer (prepared as above but with 1 mM EGTA and no added $CaCl_2$) and then for a further 5 min with an intracellular buffer prepared as described by Bader et al. (1986), composed of 140 mM L-glutamic acid, 20 mM PIPES, 6 mM Mg-acetate, 0.5 mM EGTA and 0.2% w/v bovine serum albumin, pH adjusted to 7.0 with KOH. Cells were then permeabilized in 500 μl of the same buffer but now containing 5 mM ATP and digitonin (20 μM for 6 min) or *Staphylococcus aureus* α-toxin (24 hemolytic units/ml for 30 min). The permeabilization buffer was removed and the cells stimulated with 500 μl of intracellular buffer containing 5 mM ATP, 5 mM LiCi and a buffered EGTA-Ca^{2+} concentration. After the required time the stimulation was terminated by the addition of 500 μl 30% w/v perchloric acid. [^{3}H]inositol phosphate levels in the perchloric acid extracted samples were determined as previously described by Bunn et al. (1995b). Briefly, the perchloric acid was removed with Freon and

tri-*n*-octylamine and the samples were neutralized with $NaHCO_3$ and applied to anion exchange columns (containing 1 ml of a 1:1 mix of Bio Rad AG1-X8, 100–200 mesh, formate resin and water). Following the elution of [^{3}H]inositol and [^{3}H]glycerophosphoinositol from the column with 10 ml of water and 10 ml 60 mM sodium formate, respectively, total [^{3}H]inositol phosphates (mono, bis and trisphophates) were eluted with 10 ml, 0.8 M ammonium formate in 0.1 M formic acid. The radioactive content of a 1 ml sample was measured by liquid scintillation spectrometry.

In some experiments a radioreceptor-binding mass assay, as described by Challis et al. (1991), was used to measure the levels of Ins(1,4,5)P_3 in cells that had not been previously loaded with [^{3}H]inositol. Briefly, cells were washed, stimulated and quenched with 10% perchloric acid as described above. An aliquot of the aqueous phase was then incubated for 30 min on ice in a 100 mM Tris–HCl buffer (pH 8.0) containing 4 mM EDTA, 15 μg of washed membrane protein (prepared from the bovine adrenal cortex) and approximately 10,000 dpm of [^{3}H]Ins(1,4,5)P_3. The incubation was quenched with 3 ml of ice-cold buffer as described above), filtered rapidly through GFB filters and then washed three times with a further 3 ml of ice-cold buffer. The membranes were solubilized with NCS tissue solubilizer and [^{3}H]Ins(1,4,5)P_3 was counted by liquid scintillation spectrometry. Binding was quantitated by reference to a displacement curve constructed with an unlabelled Ins(1,4,5)P_3 standard.

2.3. Fluorescence measurement of intracellular Ca^{2+}

Intracellular Ca^{2+} was monitored using the 1-[2-(5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′*N*′-tetraacetic acid pentaacetoxymethyl ester (fura-2AM) fluorescence technique as described by Zerbes et al. (1998). The glass coverslips were removed from the culture medium and the attached cells washed briefly at room temperature with 2×5 ml HEPES buffered salt solution (HSS) of the following composition NaCl 144 mM, KCl 5.4 mM, $MgCl_2$ 1.8 mM, $CaCl_2$ 1.8 mM, HEPES 10 mM, glucose 11 mM and $NaHCO_3$ 10 mM equilibrated with 100% O_2 and then adjusted to pH 7.2. The cells were loaded with fura-2 by incubating for 60 min, (in darkness), with 5 ml HSS but now containing 2 μM fura-2AM, 0.2 mg/ml pluronic F-127 and 0.2% w/v bovine serum albumin. Fura-2 loaded cells were washed for 60 min with a further 5 ml HSS before the coverslip was mounted vertically in a continuously perfused (10 ml/min HSS at 22°C) cuvette with the cells facing the light path of a Perkin-Elmer LS50B spectrofluorimeter. After a control period of perfusion with HSS for approximately 5 min the cuvette was perfused with buffers of various compositions or containing selected drugs (as indicated on the individual figures) and the fura-2 fluorescence signals recorded (alternating excitation between 340 and 380 nm and emission at 510 nm). Autofluorescence determinations were made at the end of each experiment by perfusing the cells with Ca^{2+} free HSS containing 10 mM $MnCl_2$ and ionomycin (1 μM). Data are presented as the ratio of the net fluorescence signal recorded at 340 and 380 nm excitation.

2.4. Statistical analysis

Unless otherwise stated data are presented as the means ± S.E.M. of a total of n individual observations from a specified number of separate cell preparations. All statistical analysis was performed by Student's t-test with $P < 0.05$ considered significant.

2.5. Materials

[^{3}H]*myo*-inositol and [^{3}H]Ins(1,4,5)P_3 were obtained from Amersham International (UK). Histamine, caffeine, bovine serum albumin, antibiotics and antimitotics were supplied by Sigma (St Louis, MO, USA) and nifedipine, thapsigargin and *S. aureus* α-toxin from RBI (MA, USA.). Other Ca^{2+} channel antagonists were from Alomone Labs. (Israel) and fura-2AM was from Molecular Probes (Portland, OR, USA.). Enzymes for digesting the adrenal medullae were obtained from Boehringer Mannhein (Australia) while all other cell culture reagents and media were from Cytosystems (Australia). The anion exchange resin was obtained from Bio-Rad (Australia).

3. Results

3.1. Ca^{2+} influx and phospholipase C activity

Histamine stimulated a concentration and time-dependent increase in phospholipase C activity in bovine adrenal medullary chromaffin cells. A 10-min incubation with histamine (10 μM) caused a 319 ± 11% increase in phospholipase C activity compared with cells incubated with buffer alone ($n = 9$ from three separate experiments; $P < 0.0001$). In the absence of extracellular Ca^{2+} (no added Ca^{2+} and 1 mM EGTA) there was a marked reduction in the size of the histamine-stimulated response (Fig. 1A), suggesting that a major part of this phospholipase C response is dependent upon the presence and presumably influx, of extracellular Ca^{2+}. This conclusion is supported by the observation that the inorganic Ca^{2+} channel antagonist La^{3+} (1 mM in the presence of 2.2 mM Ca^{2+}) also inhibited the phospholipase C response to histamine (Fig. 1A). It should be noted however that despite the dependency on Ca^{2+} influx histamine still stimulated a significant increase in phospholipase C activity in the absence of Ca^{2+}, or presence of La^{3+}, when compared with the response of cells under matching basal conditions (202 ± 11% $n = 9$ in three separate experiments, $P < 0.0001$, and

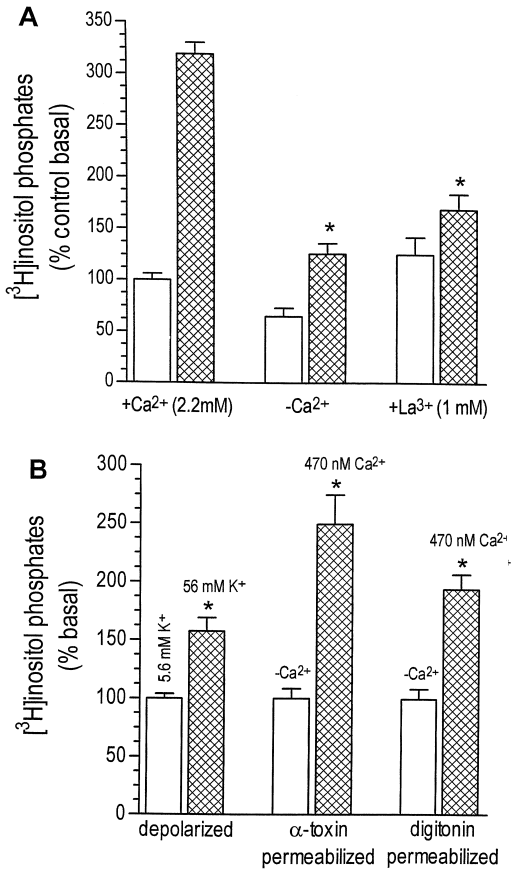

Fig. 1. The extracellular Ca^{2+} dependence of phospholipase C stimulation in bovine adrenal medullary chromaffin cells. Cells loaded with [^{3}H]inositol were stimulated for (A) 10 min with (cross hatched columns) or without (open columns) histamine (10 μM) in the continued presence or absence of extracellular Ca^{2+} (2.2 mM) or La^{3+} (1 mM) or (B) buffer with normal (5.6 mM; open column) or raised (56 mM; cross hatched column) extracellular K^+. In the permeabilization experiments in panel (B) cells were first permeabilized with α-toxin or digitonin and then incubated with Ca^{2+} free buffer (open columns) or 470 nM Ca^{2+} (cross-hatched columns) for 10 mins. All stimulations were terminated with 10% w/v perchloric acid and the [^{3}H]inositol phosphates assayed as described in Section 2. Data are presented as % control basal [^{3}H]inositol phosphates generated following stimulation with Ca^{2+} containing buffer alone in panel (A) or a Ca^{2+} free buffer for the permeabilization experiments in panel (B) and are the means ± S.E.M. from triplet determinations in three separate cell preparations. $^*P < 0.0001$ compared to corresponding basal value.

163 ± 25% $n = 9$ in three separate experiments, $P < 0.02$, respectively).

The importance of Ca^{2+} influx in supporting phospholipase C activity in chromaffin cells is further emphasized by the data presented in Fig. 1B. The opening of voltage-sensitive Ca^{2+} channels (VSCC) by K^+ depolarization (56 mM K^+ for 10 min) increased phospholipase C activity to 154 ± 12% basal ($n = 9$ from three separate experiments; $P < 0.0001$, Fig. 1B). Similarly, simply raising the Ca^{2+} concentration in cells rendered permeable to small molecules with α-toxin increased phospholipase C activity (Fig. 1B). The EC_{50} for this Ca^{2+} induced response was approximately 470 nM with a maximal stimulation (approximately 350% of that in a Ca^{2+} free buffer) occurring at approximately 1.6 μM Ca^{2+} (data not shown). Cells rendered permeable to larger molecules with digitonin also responded to raised Ca^{2+} with an increase in phospholipase C activity, although at the same Ca^{2+} concentration they were significantly less responsive than α-toxin permeabilized cells (Fig. 1B).

It was important to demonstrate that these Ca^{2+} dependent increases in phospholipase C activity actually resulted in the formation of the intracellular signalling molecule Ins(1,4,5)P_3. Incubation of permeabilized cells with 470 nM Ca^{2+} resulted in a rapid increase in Ins(1,4,5)P_3 levels which were maximal by 5 s (approximately 230% basal) and then declined slightly by 15 s before rising again by 60 s (Fig. 2). This profile suggests a biphasic Ins(1,4,5)P_3 response with an initial transient peak followed by a more sustained secondary phase. Depolarization with high K^+ (56 mM) also resulted in a rapid rise in Ins(1,4,5)P_3 levels (180% basal at 5 s) although in this case the response had returned to basal levels by 10 s (Fig. 2). The time course of Ins(1,4,5)P_3 changes in response to histamine (10 μM) was similar to that seen in permeabilized cells in response to raised Ca^{2+} (Fig. 2). The histamine response had peaked by the earliest time point examined (approximately 200% basal at 5 s) before proceeding to a secondary delayed phase which did not rise above the initial peak response (approximately 150% basal).

3.2. Characterization of the Ca^{2+} channels supporting phospholipase C activity

The nature of the channels mediating the Ca^{2+} influx supporting K^+ depolarization or histamine stimulated phospholipase C activity has been examined in more de-

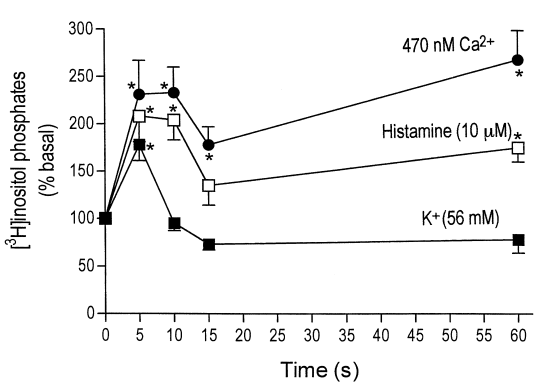

Fig. 2. Ins(1,4,5)P_3 levels in chromaffin cells after exposure to histamine, high K^+ or raised intracellular Ca^{2+}. Intact chromaffin calls were stimulated with histamine (10 μM; □) or high K^+ (56 mM; ■) and α-toxin permeabilized cells with 470 nM Ca^{2+} (●) for 5, 10, 15 or 60 s. After terminating the incubation with 10% w/v perchloric acid the mass of Ins(1,4,5)P_3 was measured using a radioligand binding assay as described in Section 2. Data are expressed as % of the basal response at each corresponding time and are the mean ± S.E.M. of 9–15 determinations from three to five separate cell preparations ($^*P < 0.002$).

tail. None of the channel blockers described below had any significant effect on basal phospholipase C activity (data not shown). The phospholipase C response to histamine was not affected by ω-agatoxin IVA (100 nM) or ω-conotoxin GVIA (300 nM), antagonists of P/Q-type and N-type Ca^{2+} channels, respectively (Fig. 3A). The histamine response was however significantly inhibited by nifedipine (10 μM) an antagonist of L-type channels (86 ± 3% of the control histamine response; $n = 15$ from 4 separate experiments; $P < 0.003$), and ω-conotoxin MVIIC (100 nM), an antagonist with limited selectivity for Q-type channels (66 ± 11% of the control response; $n = 9$ from three separate experiments; $P < 0.01$). The simultaneous addition of all of the above VSCC antagonists resulted in an inhibition similar to that produced by ω-conotoxin MVIIC alone (64 ± 5% control, $n = 12$ from four separate experiments; $P < 0.0001$). It should be noted however that even in the simultaneous presence of all VSCC antagonists the histamine response was still significantly above basal (Fig. 3A, $P < 0.0001$). The VSCC antagonists also inhibited the phospholipase C response to K^+ depolarisation although with a different profile to their effect on the histamine response (Fig. 3B). Thus, while ω-agatoxin IVA was again ineffective, ω-conotoxin GVIA produced a significant inhibition (84 ± 4% control, $n = 12$ from three separate experiments; $P < 0.005$) which, in contrast to the histamine response, was very similar to the level of inhibition produced by ω-conotoxin MVIIC (81 ± 4% control, $n = 12$ from three separate experiments; $P < 0.005$). Nifedipine, or the combination of all VSCC antagonists totally inhibited the K^+ response reducing the level of [^{3}H]inositol phosphates to those found under basal conditions. 1-[β-[3-(4-Methoxyphenyl)propoxy]-4-methoyphenethyl]-*H*-imidazole (SKF-96365), a non-selective Ca^{2+} channel antagonist, caused a concentration-dependent inhibition of histamine stimulated phospholipase C activity. At 50 μM SKF-96365 the histamine-evoked response was reduced to 74 ± 4% control ($n = 14$ from three separate experiments; $P < 0.0001$) while at 100 μM it was reduced to a level only slightly above basal. Phospholipase C activity in response to K^+ depolarization was also reduced by SKF-96365, although in this case both 50 and 100 μM resulted in complete inhibition.

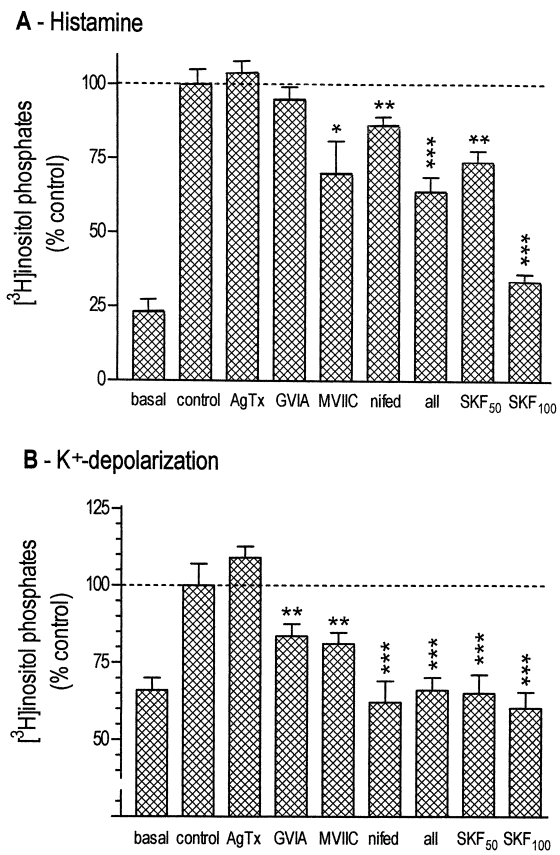

Fig. 3. The effect of channel blockers on [^{3}H]inositol phosphate generation by (A) histamine or (B) K^+ depolarization. Chromaffin cells loaded with [^{3}H]inositol were preincubated for 10 min with ω-agatoxin IVA (100 nM), ω-conotoxin GVIA (300 nM), ω-conotoxin MVIIC (100 nM), nifedipine (10 μM), a combination of all of the above Ca^{2+} channel blockers (at the previously indicated concentrations) or SKF 96365 (50 or 100 μM). The cells were then incubated for a further 10 min with histamine (10 μM ; panel (A) or K^+ (56 mM; panel (B)) in the continuing presence of the channel blockers. The stimulation was terminated and the [^{3}H]inositol phosphates assayed as described in Section 2. Data represent the mean ± S.E.M. of 9–12 determinations from three to four separate cell preparations $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0001$ compared with the control response to histamine or K^+ in the absence of any added drugs.

3.3. The intracellular Ca^{2+} signal in response to histamine

Since the influx of Ca^{2+} into chromaffin cells appeared to play a major role in regulating phospholipase C activity we investigated the nature of this Ca^{2+} signal in response to histamine using fura-2 fluorescence recording (Fig. 4). Histamine (10 μM) triggered a biphasic Ca^{2+} signal comprising an initial peak followed by a lower plateau phase which was maintained in the continued presence of histamine for at least 10 min (Fig. 4A). These two phases of the histamine Ca^{2+} signal can be separated temporally by exposing the cells to histamine in the absence of extracellular Ca^{2+} (generating a peak Ca^{2+} signal; Fig. 4B) and then, after removing the histamine stimulus with a wash in Ca^{2+} free buffer, returning the cells to a Ca^{2+} containing buffer which generated the secondary plateau phase of the Ca^{2+} response (Fig. 4B). Phospholipase C activity was measured during the two phases. During a 3 min incubation with histamine in Ca^{2+} free buffer (period 1, Fig. 4B, coinciding with the initial peak Ca^{2+} response) phospholipase C activity was increased to 142 ± 5% basal ($n = 18$ from 3 separate experiments; $P < 0.0001$, Fig. 4E). A subsequent 10 min incubation with a Ca^{2+} containing buffer (period 2 on Fig. 4B, coinciding with the plateau phase of the histamine stimulated Ca^{2+} response) did not further increase the accumulation of [^{3}H]inositol phosphates (Fig. 4E).

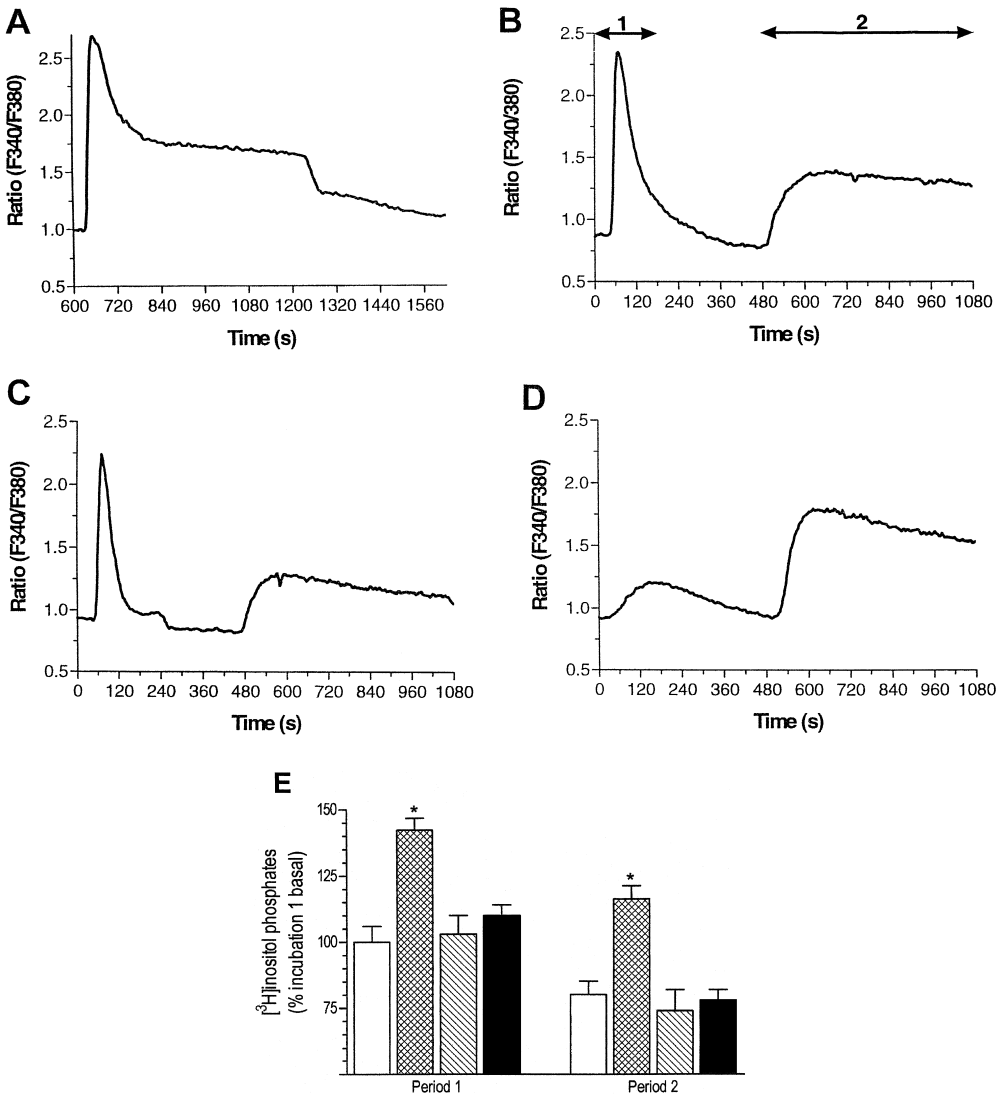

Fig. 4. The effect of capacitative Ca^{2+} influx on [^{3}H]inositol phosphate generation. Panels (A) to (D) show the fluorescent signal in chromaffin cells cultured on glass coverslips loaded with fura-2AM as described in Section 2. (A) The response to a continuous exposure to histamine (10 μM) in Ca^{2+} containing buffer. Note: the decline in the trace at approximately 1300 s is the result of washing out the histamine-containing stimulation buffer. (B) The response to histamine (10 μM for 3 min) in Ca^{2+} free buffer (stimulation period 1) followed by a 10 min wash period in Ca^{2+} free buffer before generating a capacitative Ca^{2+} influx by returning the cells to Ca^{2+} containing buffer for a further 10 min (stimulation period 2). (C) As for B but the cells were exposed to caffeine (10 mM for 3 min) during stimulation period 1. (D) As for (B) but the cells were exposed to thapsigargin (100 nM for 3 min) during stimulation period 1. The figures in panels (A) to (D) are representative of at least three similar experiments. (E) The accumulation of [^{3}H]inositol phosphates during the initial 3 min agonist-stimulation (stimulation period 1) and the subsequent capacitative Ca^{2+} entry period (stimulation period 2) for basal (open column), histamine (cross-hatched column), caffeine (shaded column) or thapsigargin (filled column) stimulated cells. Data are presented as % basal [^{3}H]inositol phosphate level generated during stimulation period 1 and are the mean ± S.E.M. of 18 determinations from three separate cell preparations ($^*P < 0.0001$ compared to the basal response over the same time period).

Biphasic Ca^{2+} responses, similar to those produced by histamine, could be generated by exposing the cells to caffeine (10 mM) or thapsigargin (100 nM). The peak response to caffeine was similar in size and duration to that of histamine (Fig. 4C) although in contrast to histamine there was no increase in phospholipase C activity during this period (Fig. 4E). Similarly, there was no increase in phospholipase C activity during the subsequent plateau phase of the caffeine response (Fig. 4E). While the peak response to thapsigargin was smaller and slower than that to the other agents the plateau generated upon returning thapsigargin treated cells to a Ca^{2+} containing buffer was notably larger (Fig. 4D). Like the caffeine response, however, there was no increase in phospholipase C activity during either the peak or plateau phases of the thapsigargin response (Fig. 4E).

4. Discussion

Receptor stimulated phospholipase C responses in chromaffin and other cell types have been described in terms of

two components, one being dependent and the other independent of Ca^{2+} influx (Sasakawa et al., 1989; Stauderman and Pruss, 1990; Wojcikiewicz and Nahorski, 1993; Bunn et al., 1995b). This report focuses on the Ca^{2+} influx dependent component of phospholipase C activation. Previous data from ourselves and others has indicated that the phospholipase C response (as measured by the accumulation of total [^{3}H]inositol phosphates) to histamine is essentially linear even in the present of 5 mM Li (Plevin and Boarder, 1988; Noble et al., 1988b; Choi et al., 1993; Bunn et al,. 1995b). Thus, depletion of phospholipase C substrate during the time course of these experiments is unlikely to be a confounding factor. In agreement with previous publications (Eberhard and Holz, 1988) we observed that increasing intracellular Ca^{2+} in permeabilized cells stimulates phospholipase C activity. Raising the Ca^{2+} concentration 10-fold from basal (about 100 nM) increased phospholipase C activity by approximately 300%. Similar results have been obtained with other permeabilized cell preparations suggesting that changes in Ca^{2+} concentration within the expected physiological range may regulate phospholipase C activity (Wojcikiewicz and Nahorski, 1993). This suggestion is supported by the demonstration by ourselves and others that the opening of VSCC by K^+ depolarization also stimulates phospholipase C in chromaffin cells (Sasakawa et al., 1989).

We show here, for the first time, that histamine stimulation, K^+ depolarization or direct elevation of intracellular Ca^{2+} in permeabilized cells, all result in apparently biphasic time courses for the formation of Ins(1,4,5)P_3 (Fig. 2). Similar biphasic Ins(1,4,5)P_3 profiles have been reported for other G-protein receptor agonists and attributed, at least in part, to receptor desensitization (Wojcikiewicz and Nahorski, 1993). Fig. 2 suggests however that the Ins(1,4,5)P_3 response to raised Ca^{2+} in permeabilized cells may also desensitize despite the presence of a constant Ca^{2+} concentration. Thus, factors downstream of receptor activation may act to modulate the cellular levels of Ins(1,4,5)P_3. This conclusion is supported by the Ins(1,4,5)P_3 time course to K^+ depolarization where Ins(1,4,5)P_3 levels were elevated only during the first few seconds despite the fact that Ca^{2+} influx to this stimulus (when measured in populations of cells), persists for at least 3 min (Zerbes et al. unpublished data).

As noted in Section 1 a number of studies have investigated the pharmacological characteristics of the Ca^{2+} channels involved in supporting histamine or K^+ depolarization induced catecholamine secretion from bovine adrenal chromaffin cells (Noble et al., 1988a; Goh and Kurosawa, 1991; O'Farrell and Marley, 1999). In this study, we provide an initial analysis of the Ca^{2+} channels involved in supporting phospholipase C activity in response to these stimuli. Previous studies have demonstrated the presence of L, N, P and Q-type VSCC on these cells (Artalejo et al., 1994; Lopez et al., 1994). A combination of antagonists sufficient to block all of these VSCC reduced the net phospholipase C response to histamine by less than 50%, while the response to K^+ depolarization was fully inhibited. The apparent involvement of VSCC in the histamine response is somewhat surprising in that histamine is reported to initially hyperpolarize rather than depolarize chromaffin cells (Artalejo et al., 1993). Nevertheless, histamine stimulated Ca^{2+} influx and catecholamine release have also been reported to be partially inhibited by dihydropyridines (Noble et al., 1988a; Goh and Kurosawa, 1991; O'Farrell and Marley, 1999). While this may indicate the participation of VSCC it is important to note that some of the antagonists used in these studies may also block non-VSCC (Willmott et al., 1996).

In agreement with previous findings concerning catecholamine secretion we found evidence for the involvement of nifedipine-sensitive L-type Ca^{2+} channels in the histamine response. It should be noted that 1 μM nifedipine inhibited only about 15% of the phospholipase C response compared to the 30–50% reported for catecholamine secretion (Noble et al., 1988a; Goh and Kurosawa, 1991; O'Farrell and Marley, 1999). ω-Conotoxin MVIIC was the only other VSCC antagonist, which significantly inhibited the histamine-induced phospholipase C response. While this toxin can inhibit most VSCC the fact that it produced a larger inhibition than nifedipine and that neither ω-agatoxin IVA or ω-conotoxin GIVA had any effect suggests that Q-(but not P- or N-type) channels may be the major non-L-type VSCC supporting the histamine response. In contrast to this histamine response, antagonism of N-type Ca^{2+} with ω-conotoxin GVIA resulted in a reduction in the size of the K^+ depolarization induced phospholipase C response.

The recent study by O'Farrell and Marley (1999) found evidence for the involvement of both ω-agatoxin IVA and ω-conotoxin-GVIA sensitive Ca^{2+} channels in supporting histamine induced catecholamine secretion from these cells. Interestingly, they found no evidence for the involvement of these channels in supporting histamine-induced activation of tyrosine hydroxylase, a response which is known to be dependent upon Ca^{2+} influx. The suggestion made by O'Farrell and Marley that this observation may indicate that tyrosine hydroxylase activation requires less Ca^{2+} than does the secretory response may also be relevant to the current data. In permeabilized cells low concentrations of Ca^{2+} are more effective at activating phospholipase C than they are at evoking secretion (data presented here and Eberhard and Holz, 1988). Thus, a partial blockade of a VSCC by a particular antagonist may reduce Ca^{2+} influx to a point at which secretion but not phospholipase C activity is inhibited. Alternatively, the differing sensitivities of histamine-induced secretion and phospholipase C activity to VSCC antagonists may indicate the involvement of different populations of Ca^{2+} channels in these two responses.

The non-selective Ca^{2+} channel antagonist SKF 96365 blocked the histamine phospholipase C response resistant

to all VSCC antagonists. This may suggest the involvement of the so-called ''capacitative'' Ca^{2+} influx pathway (Putney and McKay, 1999; Barritt, 1999) but it should be noted that relatively high concentrations of SKF 96365 were required. A 10-fold lower concentration of the drug was shown to inhibit acetylcholine-induced $^{45}Ca^{2+}$ influx and catecholamine release in these cells (Tachikawa et al., 1994). Furthermore, in the current studies we found that the phospholipase C response to K^+ depolarization was in fact more sensitive to SKF 96365 than was the histamine response. Thus, histamine stimulated phospholipase C activation may result in part from Ca^{2+} influx through a novel channel type. This suggestion is supported by two further observations in bovine adrenal chromaffin cells, firstly histamine evokes a rapid, nifedipine resistant, Ca^{2+} influx independent of capacitative or store-depletion driven Ca^{2+} entry (Cheek et al., 1994), and secondly the plateau phase of the Ca^{2+} signal to histamine cannot be fully accounted for by VSCC or capacitative entry (Zerbes et al., 1998).

Nevertheless, capacitative Ca^{2+} influx in response to histamine and other agents is clearly demonstrated in Fig. 4. When intracellular Ca^{2+} stores are depleted following exposure to histamine, caffeine or thapsigargin in Ca^{2+} free buffer a sustained Ca^{2+} influx is generated when extracellular Ca^{2+}-is returned, eventhough the original agonist is no longer present. This Ca^{2+} influx mechanism has been extensively described by others (Putney and Mckay, 1999; Barritt, 1999). What is of particular interest here is that this capacitative Ca^{2+} influx appears to be an insufficient signal for stimulating phospholipase C activity. No increase in phospholipase C activity was associated with this phase of the Ca^{2+} signal to any of the agonists, including histamine. While it should be noted that unlike the data presented elsewhere in this report these experiments did not include Li in the incubation buffer it would seem unlikely that this is a confounding factor. These data suggest either that receptor occupancy as well as Ca^{2+} influx is required for phospholipase C activity or, that the Ca^{2+} influx important for phospholipase C activation is not that which occurs through capacitative Ca^{2+} channels. The second possibility is supported by the data presented here from permeabilized cells in which phospholipase C is stimulated by Ca^{2+} elevation in the presumed absence of receptor activation. Thus, in permeabilized cells Ca^{2+} may have access to phospholipase C, which is perhaps not shared by Ca^{2+} entering the cell via capacitative Ca^{2+}-channels. This lack of involvement of capacitative Ca^{2+}-channels in supporting histamine stimulated phospholipase C activity is supported by our data indicating that the residual, non-voltage sensitive, Ca^{2+} influx appears to be relatively insensitive to SKF 96365.

The data presented in this manuscript provide the first pharmacological characterisation of the Ca^{2+} channels responsible for supporting histamine and K^+ depolarization induced phospholipase C activity in chromaffin cells. Two important conclusions can be reached. Firstly, in agreement with previous reports examining catecholamine secretion, our data indicate that different Ca^{2+} channels are activated by these two stimuli. Secondly, comparison of the data presented here for phospholipase C activation with that published previously for histamine-induced secretion suggests differences in the Ca^{2+} signal needed for these two histamine responses. It remains to be resolved as to whether this reflects the involvement of different Ca^{2+} channels or differences in the absolute levels of Ca^{2+} required.

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References

Artalejo, A.R., Garcia, A.G., Neher, E., 1993. Small-conductance Ca^{2+} activated K^+ channels in bovine chromaffin cells. Pfluegers Arch. 423, 97–103.

Artalejo, C.R., Adams, M.E., Fox, A.P., 1994. Three types of Ca^{2+} channel trigger secretion with different efficacies in chromaffin cells. Nature 367, 72–76.

Bader, M.F., Thierse, D., Aunis, D., Ahnert-Hilger, G., Gratzl, M., 1986. Characterization of hormone and protein release from α-toxin-permeabilized chromaffin cells in primary culture. J. Biol. Chem. 261, 5777–5783.

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.

Bunn, S.J., Boyd, T.L., 1992. Characterization of histamine-induced catecholamine secretion from bovine adrenal medullary chromaffin cells. J. Neurochem. 58, 1602–1610.

Bunn, S.J., Dunkley, P.R., 1997. Histamine-stimulated phospholipase C signalling in the adrenal chromaffin cell: effects on inositol phospholipid metabolism and tyrosine hydroxylase phosphorylation. Clin. Exp. Pharmacol. Physiol. 24, 624–631.

Bunn, S.J., Marley, P.D., Livett, B.G., 1990. Receptor stimulated formation of inositol phosphates in cultures of bovine adrenal medullary cells: the effects of bradykinin, bombesin and neurotensin. Neuropeptides 15, 187–194.

Bunn, S.J., Sim, A.T.R., Herd, L.M., Austin, L.M., Dunkley, P.R., 1995a. Tyrosine hydroxylase phosphorylation in bovine adrenal chromaffin cells: the role of intracellular Ca^{2+} in the histamine H_1 receptor-stimulated phosphorylation of ser^8, ser^{19} ser^{31}, and ser^{40}. J. Neurochem. 64, 1370–1378.

Bunn, S.J., Saunders, H.I., Dunkley, P.R., 1995b. Histamine-stimulated inositol phospholipid metabolism in bovine adrenal medullary cells: a kinetic analysis. J. Neurochem. 65, 626–635.

Challis, 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., Morgan, A., O'Sullivan, A.J., Moreton, R.B., Berridge, M.J., Burgoyne, R.B., 1993. Spatial localization of agonist-induced Ca^{2+} entry in bovine adrenal chromaffin cells: different patterns induced by histamine and angiotensin II, and relationship to catecholamine release. J. Cell Sci. 105, 913–921.

Cheek, T.R., Murawsky, M.M., Stauderman, K.A., 1994. Histamine-induced Ca^{2+} entry precedes Ca^{2+} mobilization in bovine chromaffin cells. Biochem. J. 304, 469–476.

Choi, A.Y., Cahill, A.L., Perry, B.D., Perlman, R.L., 1993. Histamine evokes greater increases in phosphatidylinositol metabolism and catecholamine secretion in epinephrine-containing than in norepinephrine-containing chromaffin cells. J. Neurochem. 61, 541–549.

Eberhard, D.A., Holz, R.W., 1988. Intracellular Ca^{2+} activates phospholipase C. Trends Neurosci. 11, 517–520.

Firestone, J.A., Browning, M.D., 1994. Calcium signalling in bovine adrenal chromaffin cells: additive effects of histamine and nicotine. Synapse 17, 268–274.

Goh, Y., Kurosawa, A., 1991. Characterization and Ca^{2+} requirements of histamine-induced catecholamine secretion in cultured bovine chromaffin cells. J. Neurochem. 57, 1249–1257.

Lee, S.B., Rhee, S.G., 1995. Significance of PIP_2 hydrolysis and regulation of phospholipase C isozymes. Current Opin. Cell Biol. 7, 183–189.

Livett, B.G., Marley, P.D., 1986. Effects of opioid peptides and morphine on histamine-induced catecholamine secretion from culture, bovine adrenal chromaffin cells. Br. J. Pharmacol. 98, 327–334.

Lopez, M.G., Villarroya, M., Lara, B., Sierra, R.M., Albillos, A., Garcia, A.G., Gandia, L., 1994. Q- and L-type channels dominate the control of secretion in bovine chromaffin cells. FEBS Lett. 349, 331–337.

Noble, E.P., Bommer, M., Liebisch, D., Herz, A., 1988a. H_1-histaminergic activation of catecholamine release by chromaffin cells. Biochem. Pharmacol. 37, 221–228.

Noble, E.P., Bommer, M., Sincini, E., Costa, T., Herz, A., 1988b. H_1-histaminergic activation stimulates inositol-1-phosphate accumulation in chromaffin cells. Biochem. Biophys. Res. Commun. 135, 566–573.

O'Farrell, M., Marley, P.D., 1999. Different contributions of voltage-sensitive Ca^{2+} channels to histamine-induced catecholamine release and tyrosine hydroxylase activation in bovine adrenal chromaffin cells. Cell Calcium 25, 209–217.

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 carbachol. J. Neurochem. 51, 634–641.

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.

Putney, J.W., McKay, R.R., 1999. Capacitative calcium entry channels. BioEssays 21, 38–46.

Robinson, I.M., Burgoyne, R.D., 1991. Characterisation of distinct inositol 1,4,5-trisphosphate-sensitive and caffeine-sensitive calcium stores in digitonin-permeabilised adrenal chromaffin cells. J. Neurochem. 56, 1587–1593.

Sasakawa, N., Nakaki, T., Yamamoto, S., Kato, R., 1989. Calcium uptake-dependent and independent mechanisms of inositol trisphosphate formation in adrenal chromaffin cells: comparative studies with high K^+, carbamylcholine and angiotensin II. Cell Signalling 1, 75–84.

Singer, W.D., Brown, H.A., Sternweis, P.C., 1997. Regulation of eukaryotic phosphatidylinositol-specific phospholipase C and phospholipase D. Annu. Rev. Biochem. 66, 475–509.

Stauderman, K.A., Pruss, R.M., 1990. Different patterns of agonist-stimulated increases of ^{3}H-inositol phosphate isomers and cytosolic Ca^{2+} in bovine adrenal chromaffin cells: comparison of the effects of histamine and angiotensin II. J. Neurochem. 54, 946–953.

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.

Willmott, N.J., Choudhury, Q., Flower, R.J., 1996. Functional importance of the dihydropyridine-sensitive, yet voltage-insensitive store-operated Ca^{2+} influx of U937 cells. FEBS Lett. 394, 159–164.

Wojcikiewicz, R.J.H., Nahorski, S.R., 1993. Modulation of signalling initiated by phosphoinositidase-C-linked receptors. J. Exp. Biol. 184, 145–159.

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.

Zhang, L., Del Castillo, A.R., Trifaro, J.M., 1995. Histamine-evoked chromaffin cell scinderin redistribution, F-actin disassembly, and secretion: in the absence of cortical F-actin disassembly, an increase in intracellular Ca^{2+} fails to trigger exocytosis. J. Neurochem. 65, 1297–1308.