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Research report

Vasoactive intestinal peptide potentiates and directly stimulates catecholamine secretion from rat adrenal chromaffin cells

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Abstract

The actions of vasoactive intestinal polypeptide (VIP) on catecholamine secretion and changes in $[Ca^{2+}]_i$ in single rat chromaffin cells were studied using amperometry and Indo-1. Application of VIP prior to acetylcholine (ACh) or co-application of VIP and ACh enhanced secretion by 94% and 153% respectively, compared to ACh alone. $[Ca^{2+}]_i$ was increased by 17% when VIP was preapplied and by 73% upon co-application. Exposure to VIP before stimulation with 60 mM K^+ enhanced secretion by 68%, but not $[Ca^{2+}]_i$. VIP application prior to DMPP and nicotine had no effect on $[Ca^{2+}]_i$, but increased $[Ca^{2+}]_i$ signals to muscarine by 18%. VIP co-application potentiated only $[Ca^{2+}]_i$ responses to muscarine, by 28%. The effect of VIP on muscarine-induced $[Ca^{2+}]_i$ signals was mimicked by 8-Br-cAMP, and both were blocked by H-89, a protein kinase A inhibitor. Long-lasting increases in secretion accompanied by a sustained rise in $[Ca^{2+}]_i$ to VIP alone were seen in 55% of cells. Removal of Ca^{2+} or addition of La^{3+} inhibited both responses, while L-, N- and P-type Ca^{2+} channel blockers were ineffective. SK&F 96365 inhibited VIP-induced secretion completely and rises in $[Ca^{2+}]_i$ by 75%. Neither 8-Br-cAMP nor 8-Br-cGMP evoked responses similar to VIP alone. Thus in rat chromaffin cells, VIP acts both directly as a neurotransmitter in provoking sustained catecholamine secretion in a cAMP-independent manner, and also by enhancing ACh-induced secretion, via a cAMP-dependent action involving muscarinic receptors.

Keywords: Rat; Adrenal chromaffin cell; Catecholamine release; Intracellular calcium; cAMP

1. Introduction

Vasoactive intestinal polypeptide (VIP) is a 28-amino acid peptide first isolated from porcine small intestine which acts as a neurotransmitter released from noradrenergic and non-cholinergic nerve terminals in a variety of organs and tissues. It participates in the control of smooth muscle tone and motility, blood flow and secretion [12,35]. As well, VIP co-exists with ACh in neurons from rat cerebral cortex, in postganglionic parasympathetic nerves supplying exocrine glands and in splanchnic nerve terminals in the adrenal medulla [10,18,25,40].

In the adrenal medulla, VIP functions as a neurotransmitter and/or as a modulator of catecholamine secretion [38]. Infusion of VIP into adrenal glands from rat [26–28] and calves [11] results in catecholamine release similar to the non-cholinergic component observed upon nerve stimulation. Such direct stimulatory effects have also been observed for suspensions of chromaffin cells from guinea pig [31] and in single rat chromaffin cells [7]. In contrast, the effects of VIP on $[Ca^{2+}]_i$ in chromaffin cells are less clear. It has been reported that VIP-associated secretion is not accompanied by an increase of $[Ca^{2+}]_i$ [31] or $^{45}Ca^{2+}$ uptake [27] and that removal of external Ca^{2+} is without effect [26,27,31]. On the other hand, pituitary adenylate cyclase-activating polypeptide (PACAP), which shares substantial homology with VIP and often has similar functional effects [17], causes significant elevation of $[Ca^{2+}]_i$ in rat [34,39], porcine [21] and frog [44] chromaffin cells, associated with stimulation of catecholamine secretion.

Because a rise in $[Ca^{2+}]_i$ is an essential factor for exocytosis in chromaffin cells [4], we focused on the relation between VIP-evoked secretion and $[Ca^{2+}]_i$. So far, all single-cell studies of the effects of VIP on these two parameters have been carried out independently. Thus, in most experiments, we measured simultaneously cate-

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cholamine release using carbon fibre-based microelectrodes [6,7,24] and $[Ca^{2+}]_i$ using Indo-1 in single rat chromaffin cells. Our results show that VIP enhances secretion evoked by ACh, and also directly stimulates long-lasting secretion accompanied by a sustained increase of $[Ca^{2+}]_i$.

2. Materials and methods

2.1. Cell culture

Chromaffin cell cultures were prepared from Wistar rats as previously described [37]. Cells were plated on thin glass coverslips coated with polyornithine and used within 3–10 d.

2.2. $[Ca^{2+}]_i$ measurements

Indo-1 fluorescence was measured on an inverted microscope (Nikon Diaphot) using a 40× UV-Fluor oil immersion objective (n.a. 1.3; Nikon). Excitation light from a 100 W Xe arc lamp (Osram) was limited using neutral density filters (ND 16 or 32) to minimize bleaching, bandpass filtered at 360 ± 5 nm and reflected off a dichroic mirror (380 nm). Emitted epifluorescence was limited to a single cell by an adjustable rectangular diaphragm, passed to another dichroic mirror (455 nm), and after bandpass filtering at 405 ± 10 and 480 ± 10 nm, intensities at both wavelengths were recorded simultaneously using two photomultipliers with matched photometers (P1, Nikon). The ratio ($R = F_{405}/F_{480}$), reflecting $[Ca^{2+}]_i$, was calculated on-line, displayed on a monitor, sampled at 20 Hz and stored in a computer for later analysis. Cells were loaded by incubation for 10 min in standard external solution (in mM: 145 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES, 10 glucose, pH 7.3 with NaOH) containing 5 μM Indo-1/AM and 0.02% Pluronic F-127 (Molecular Probes) at 37°C in the incubator. Cells were then washed twice and allowed to rest at room temperature for 10 min before use. Cells were viewed in a thin (0.07 mm), glass-bottomed Petri dish in the dark or under dim light at room temperature (22–25°C). Changes in $[Ca^{2+}]_i$ and secretion were always simultaneously measured from single cells, except for the data presented in Figs. 3 and 4.

2.3. Measurement of catecholamine release

Carbon fibre microelectrodes were used to detect amperometrically [6,7,24] release of catecholamines from single Indo-1-loaded, rat chromaffin cells. Electrodes were made by inserting a 2-cm long carbon fibre (7 or 33 μm diameter) into a patch pipette. In some cases, carbon fibres were insulated with a thin plastic coating [6] obtained by heating and pulling down by hand ≈1 mm diameter

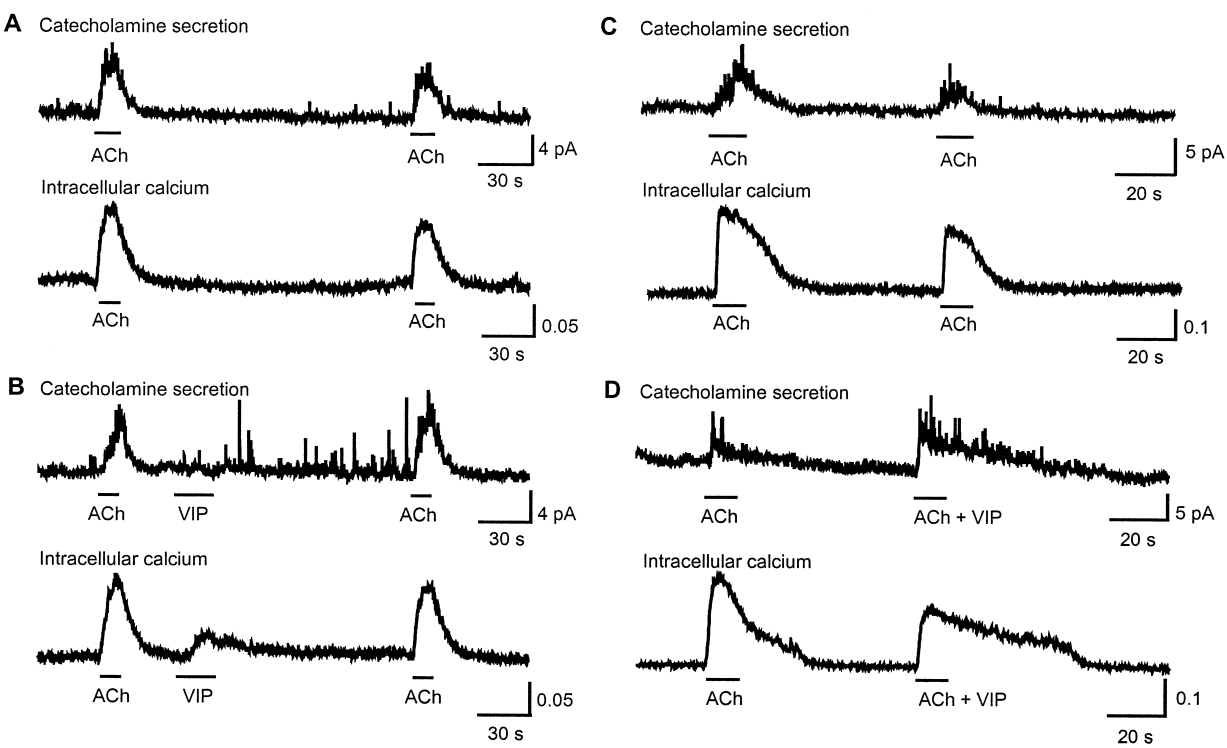

Fig. 1. Effects of VIP on ACh-evoked catecholamine secretion and changes in $[Ca^{2+}]_i$ simultaneously measured in single rat chromaffin cells. (A) Control responses for two 10-s applications of 1 μM ACh separated by ≈170 s. (B) Effect of 10 μM VIP applied for 20 s before a second challenge with 1 μM ACh 130 s later. (C) Control responses for two 10-s applications of 1 μM ACh separated by ≈60 s. (D) Effects of 10 μM VIP co-applied with 1 μM ACh.

plastic tubing made from pipetteman yellow tips. The fibre was sealed into the pipette with cyanoacrylate glue or beeswax and trimmed to an exposed length of ≤ 10 μm. The pipette was back-filled with 3 M KCl and connected to an amplifier (List EPC-7) with a Ag/AgCl wire; the bath reference electrode was a Ag/AgCl wire. Electrode resistances were $> 2–5$ GΩ and sensitivity was routinely monitored before and after experiments using 0.001–10 μM adrenaline and fast cyclic voltametry (not shown). Catecholamine oxidation currents were measured at 0.65 V (i.e., constant potential amperometry) from single cells with the electrode positioned close to the cell (≈ 1 μm away). Lack of response to different applied substances in the absence of cells was verified. High (60 mM) K^+ external solution was made by substitution with NaCl; Ca^{2+}-free external solution had no added Ca^{2+} and contained 1 mM EGTA. Secretagogues were applied, usually for 10 or 20 s, using separate puffer pipettes. Secretion and changes in $[Ca^{2+}]_i$ were assessed by calculating the area below the curves until they returned to baseline. These values were expressed in percentages, with 100% corresponding to the initial response. The bath (volume ≈ 1.5 ml) was perfused at a low rate (0.2 ml/min) during recording to minimize noise. Data are given as mean ± S.D. for *n* cells where appropriate. Statistical significance was evaluated using ANOVA and post-testing with the Mann–Whitney *U*-test; Bonferroni's correction for multiple comparisons to a single value was applied.

2.4. Materials

Acetylcholine, muscarine, nicotine and forskolin were from Sigma; DMPP {1,1 dimethyl-4-phenylpiperazinium iodide} was from Aldrich; VIP was from Neosystem (Strasbourg); nifedipine, ω-conotoxin GVIA (ωCgTx), H-89 {*N*-(2-((3-(4-bromo-phenyl))-2-propenyl)-amino)-ethyl))-5 isoquinoline sulfonamide dihydro-chloride} and SK & F 96365 {1-[β-3[-(4-methoxy-phenol)propoxyl]-4-methoxy-phenethyl]-1*H*-imidazole, HCl} were from Calbiochem; ω-agatoxin IVA (AgaIVa) was from Peptide Institute; 8-Br-cAMP and 8-Br-cGMP were from Boehringer.

3. Results

3.1. VIP potentiates ACh- and K^+-evoked catecholamine release

To evaluate the effects of VIP on secretion from rat chromaffin cells, the stability of catecholamine release and

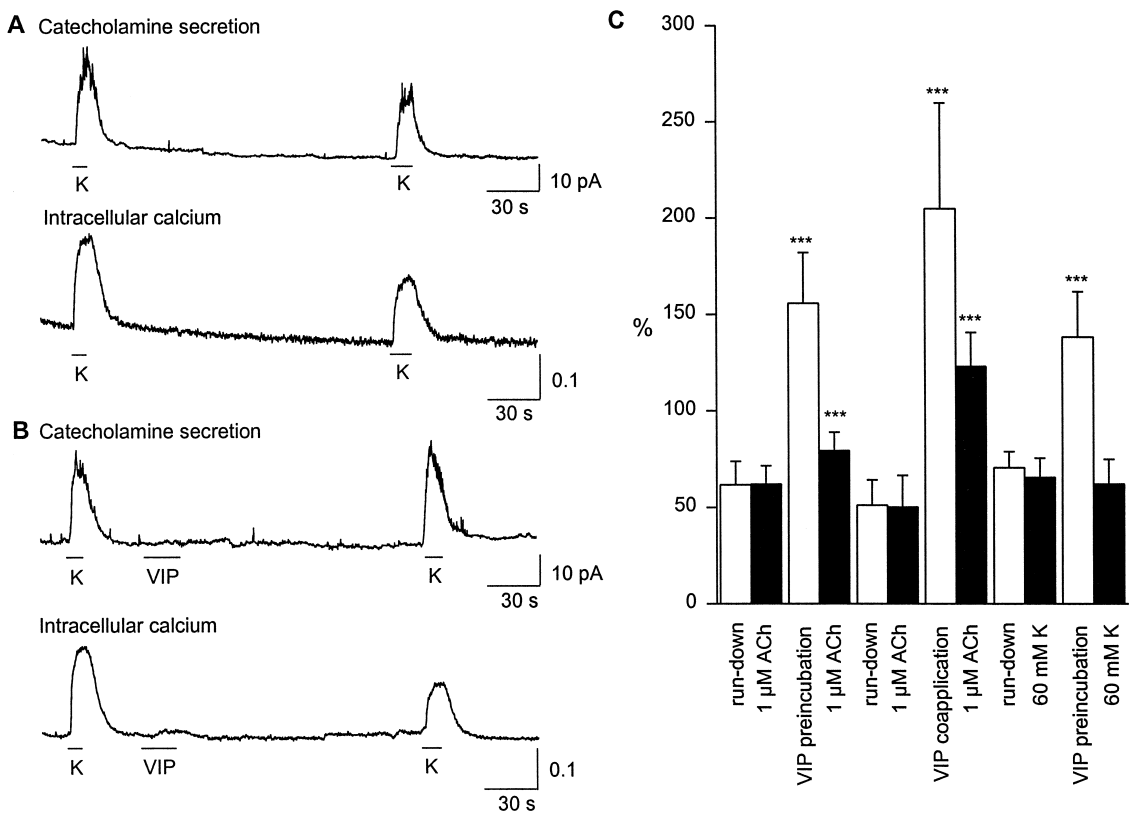

Fig. 2. Effects of VIP on 60 mM K^+-evoked responses. (A) Control responses for two 10-s applications of 60 mM K^+ solution separated by 170 s. (B) Effect of 10 μM VIP applied for 20 s before a second 60 mM K^+ challenge 130 s later. (C) Average data summarizing the effects of 10 μM VIP pre- or co-applied on secretagogue-induced changes in secretion (open columns) and $[Ca^{2+}]_i$ (filled columns). Rundown for the second responses to each secretagogue alone are also shown. Note that 100% corresponds to the initial responses evoked by 1 μM ACh or 60 mM K^+ alone. Error bars represent ± S.D.; $***$ $p < 0.001$ (compared to control rundown values).

changes in $[Ca^{2+}]_i$ to two successive 10-s applications of 1 μM ACh (separated by 170 ± 10 s) was first estimated (Fig. 1A). For the second ACh application, secretion and rises in $[Ca^{2+}]_i$ were both reduced by ~40% on average (Fig. 2C; $n = 17$), compared to initial responses. When 10 μM VIP was applied for 10–20 s before the second challenge with ACh (Fig. 1B), secretion was greatly increased with the rise in $[Ca^{2+}]_i$ being also slightly potentiated. Taking into account the average rundown of ~40% observed for successive ACh applications (Fig. 1A), preapplication of VIP-potentiated secretion induced by a subsequent challenge by ACh by 94%, with $[Ca^{2+}]_i$ rises being increased by ~20% (Fig. 2C; $n = 16$), compared to the initial ACh-induced responses.

A much larger potentiating effect of 10 μM VIP on both secretion and rises in $[Ca^{2+}]_i$ was seen upon co-application with 1 μM ACh (Fig. 1D). In this series of experiments, control responses for a second successive application of ACh alone (Fig. 1C) were both reduced on average by ~50% (Fig. 2C; $n = 15$), compared to the first challenge with ACh. Taking into account this rundown, co-application of VIP with ACh enhanced secretion by 153% and rises in $[Ca^{2+}]_i$ by 73% (Fig. 2C; $n = 18$), compared to ACh alone. Furthermore, co-application of VIP also clearly increased the duration of both signals (Fig. 1C,D).

The effects of VIP on responses to 60 mM K^+ were studied in order to assess possible modulation of voltage-dependent Ca^{2+} channels by VIP. The rundown of secretion and $[Ca^{2+}]_i$ elevation for a second application of high K^+, 170 ± 10 s after a first stimulus, was estimated (Fig. 2A): secretion was reduced by ~30% and rises in $[Ca^{2+}]_i$ by ~35% on average (Fig. 2C; $n = 28$). When 10 μM VIP was applied before the second high K^+ stimulation, secretion but not rises in $[Ca^{2+}]_i$ were increased compared to the initial responses to K^+ (Fig. 2B). Taking into account rundown, VIP increased K^+-induced secretion by 68% while changes in $[Ca^{2+}]_i$ were unaffected (Fig. 2C; $n = 15$). Co-application of VIP with 60 mM K^+ did not affect secretion or $[Ca^{2+}]_i$ signals (not shown), compared to high K^+ alone. This suggests that VIP does not modulate voltage-dependent Ca^{2+} channel activity.

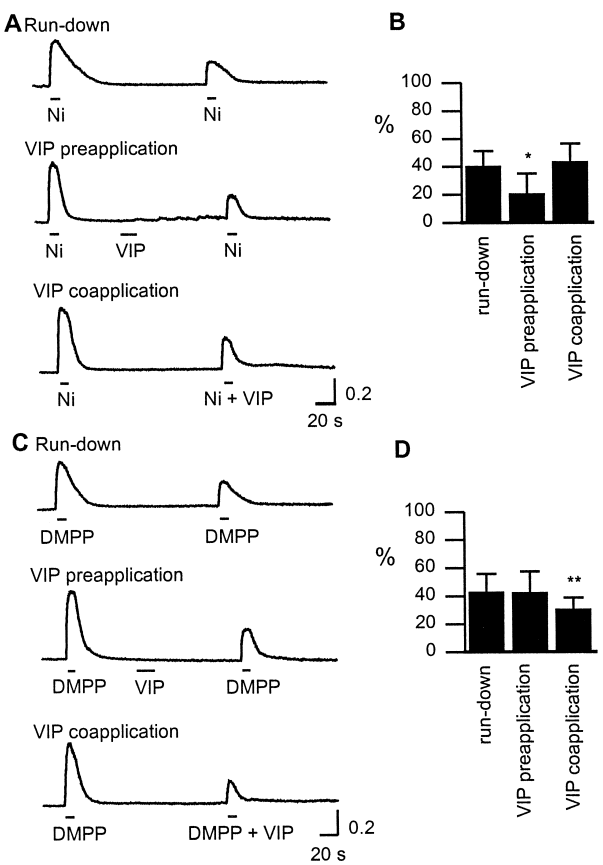

Fig. 3. Effect of VIP on nicotine- and DMPP-induced changes in $[Ca^{2+}]_i$. Neither prior nor co-application of 10 μM VIP cause potentiation of second $[Ca^{2+}]_i$ responses evoked by 10 μM nicotine (A) or 10 μM DMPP (C). Average data for second responses to nicotine (B) and DMPP (D); 100% corresponds to the initial response values; $* p < 0.05$; $** p < 0.01$ (compared to control rundown).

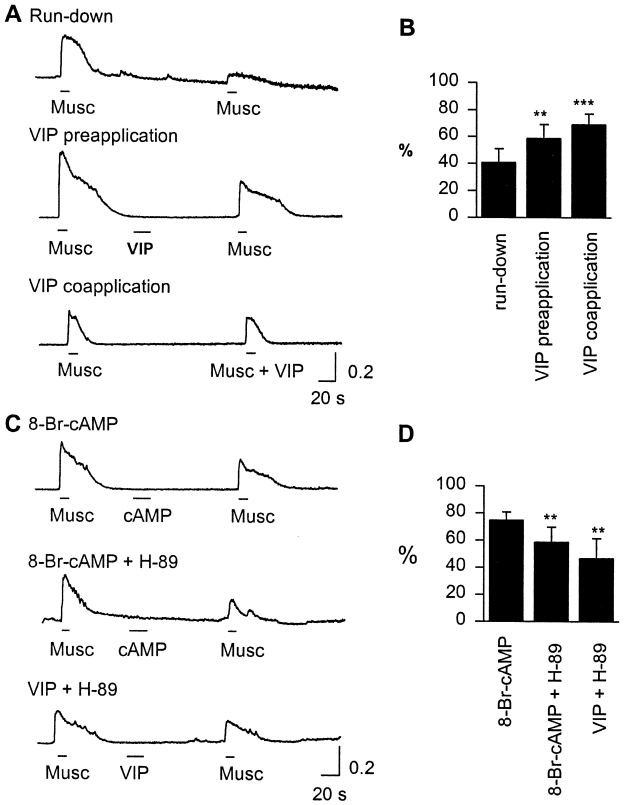

Fig. 4. Effects of VIP and 8-Br-cAMP on muscarine-induced changes in $[Ca^{2+}]_i$. (A) Both prior and co-application of 10 μM VIP increase the second 100 μM muscarine-evoked $[Ca^{2+}]_i$ signals. Average data are given in (B); $** p < 0.01$; $*** p < 0.001$ (compared to control rundown). (C) Prior application of 50 μM 8-Br-cAMP increases the second 100 μM muscarine-induced $[Ca^{2+}]_i$ signal. The potentiating effect of both 8-Br-cAMP and VIP are abolished by 20-min preincubation with 300 nM H-89. Average data are given in (D); $** p < 0.01$ (compared to control rundown).

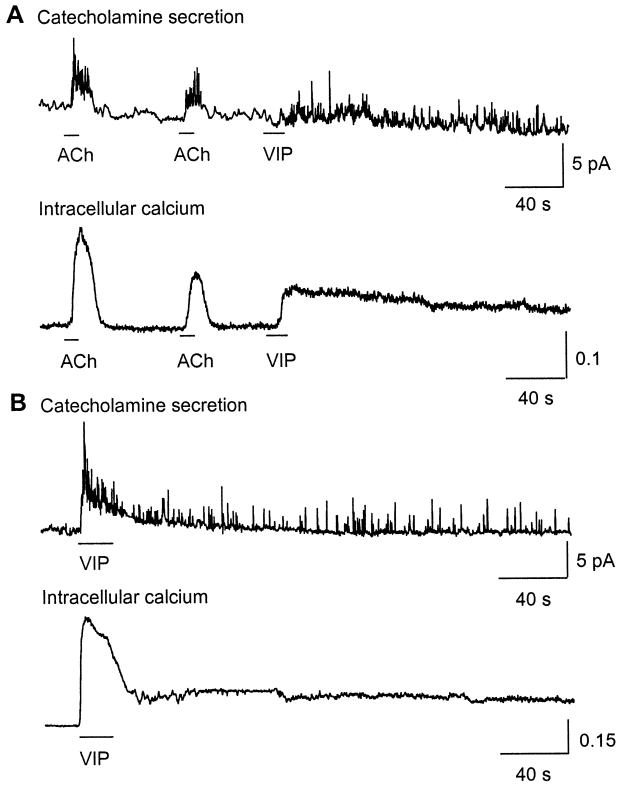

Fig. 5. The VIP acts as a secretagogue in rat chromaffin cells. (A) Simultaneously measured responses for 20-s applications of 1 μM ACh and 10 μM VIP. (B) Another cell showing the long-lasting effects of 10 μM VIP.

3.2. VIP potentiates muscarine-induced increases in $[Ca^{2+}]_i$

Since Ca^{2+} channels were apparently not affected by VIP, the involvement of nicotinic and muscarinic receptors in the potentiating effect of VIP on ACh-induced $[Ca^{2+}]_i$ signals was assessed. For a second application of 10 μM nicotine (Fig. 3A,B; $n = 10$), 10 μM DMPP (Fig. 3C,D; $n = 28$) and 100 μM muscarine (Fig. 4A,B; $n = 16$), rises in $[Ca^{2+}]_i$ were all reduced on average by ~60%, compared to initial agonist-induced responses. When 10 μM VIP was applied prior to the second agonist challenge, $[Ca^{2+}]_i$ signals induced by nicotine were decreased by ~50% on average (Fig. 3A,B; $n = 8$) while those produced by DMPP were unaffected (Fig. 3C,D; $n = 8$). In contrast, only muscarine-evoked changes in $[Ca^{2+}]_i$ were potentiated (by 18%; Fig. 4A,B; $n = 7$) by VIP preapplication. Much the same pattern was seen upon co-application of VIP, with only $[Ca^{2+}]_i$ signals to muscarine being enhanced on average (by 28%; Fig. 4A,B; $n = 5$); nicotine-induced responses ($n = 10$) were unchanged and those for DMPP were reduced ($n = 11$). In agreement, inward whole-cell currents activated by 100 μM nicotine were unaffected by 10 μM VIP in amphotericin-B, perforated patch recordings ($n = 4$; not shown). As VIP elevates cAMP in chromaffin cells [28,33,41], the stimulatory effect of VIP on muscarine-induced $[Ca^{2+}]_i$ signals may involve cAMP. Compared to control (rundown to ~40% of initial responses; Fig. 4B), preapplication of 50 μM 8-Br-cAMP increased $[Ca^{2+}]_i$ signals by ~35% ($n = 7$; Fig. 4D) for a second muscarine challenge. This stimulatory effect of 8-Br-cAMP was abolished by 20 min preincubation with 300 nM H-89, a selective protein kinase A inhibitor [5], with the second muscarine-induced $[Ca^{2+}]_i$ signals ($n = 8$) being not different from control. A similar reduction of the VIP stimulatory effect was seen with H-89: the second $[Ca^{2+}]_i$ signals for muscarine (Fig. 4C,D; $n = 7$) were reduced compared to those in the absence of H-89 (Fig. 4A,B). Thus, the potentiating effect of VIP on ACh-stimulated rises in $[Ca^{2+}]_i$ involves muscarinic receptors and the cAMP/PKA pathway.

3.3. VIP directly evokes long-lasting increases in $[Ca^{2+}]_i$ and catecholamine secretion

Although VIP alone was without effect in many cells, 10 μM VIP triggered long-lasting release and a sustained increase in $[Ca^{2+}]_i$, both with a rapid onset (Fig. 5A), in 55 of 100 cells. In contrast, responses evoked by 1 μM

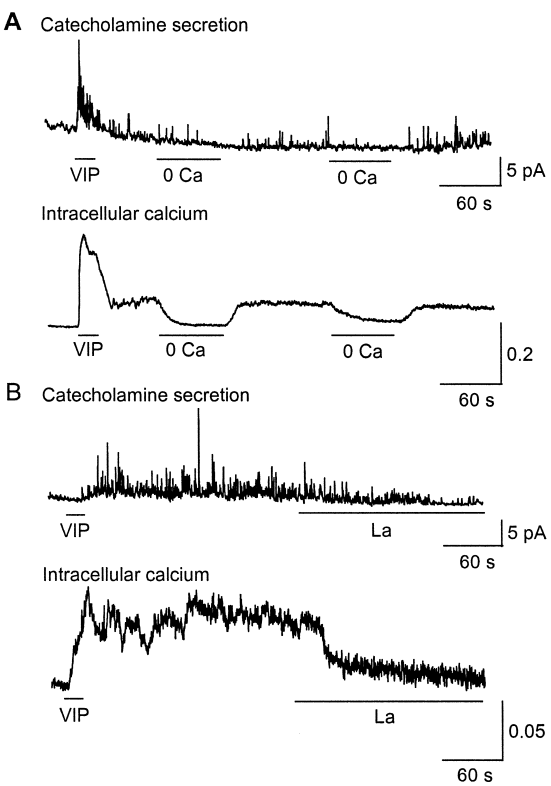

Fig. 6. The VIP-induced secretion and rises in $[Ca^{2+}]_i$ depend on external Ca^{2+}. (A) Effects of removal of external Ca^{2+} on VIP-induced secretion and changes in $[Ca^{2+}]_i$ in the same cell. After 10 μM, VIP was applied for 20 s in standard external solution (with 2 mM Ca^{2+}), the cell was then locally microperfused twice for 1 min with Ca^{2+}-free external solution (separate puffer pipettes were used). (B) Effect of bath application of 100 μM La^{3+} on responses evoked by 10 μM VIP.

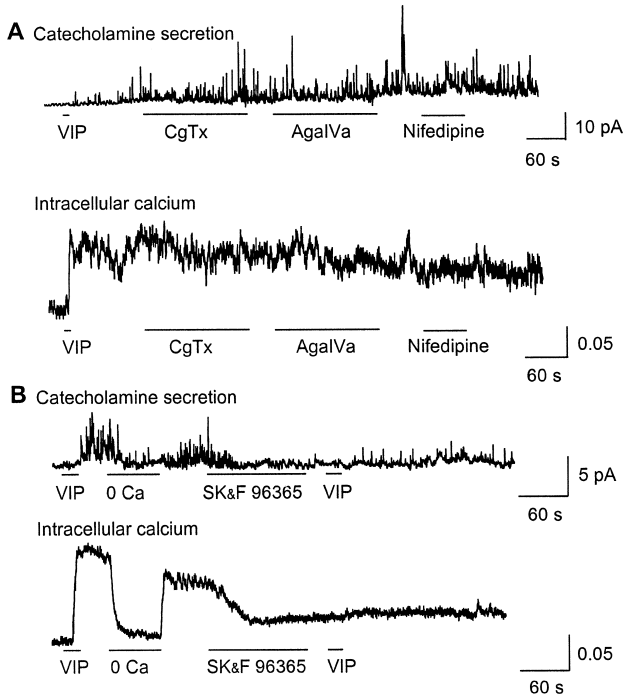

Fig. 7. Effects of Ca^{2+}-entry blockers on VIP-induced responses. (A) Effect of Ca^{2+} channel blockers 1 μM ω-conotoxin GVIA (CgTx), 200 nM agatoxin IVa (AgaIVa) and 1 μM nifedipine on secretion and elevation of $[Ca^{2+}]_i$ produced by 10 μM VIP in the same cell. (B) Effect of 20 μM SK&F 96365 on secretion and increase in $[Ca^{2+}]_i$ induced by 10 μM VIP. Antagonists were applied using separate puffer pipettes also containing 10 μM VIP.

ACh (Fig. 5A) were much briefer. In cells where ACh was not applied before VIP, secretion and the elevation in $[Ca^{2+}]_i$ were distinctly biphasic with an initial transient followed by a plateau (Fig. 5B; $n = 12$). This difference in form may depend on whether internal Ca^{2+} stores were full: VIP-induced rises in $[Ca^{2+}]_i$ after muscarine were never biphasic ($n = 10$), unlike after DMPP, when they were always biphasic ($n = 5$). Further increases in VIP had no stronger effects on secretion or changes in $[Ca^{2+}]_i$, while at 1 μM, VIP had only weak or negligible effects on secretion (not shown).

The VIP-induced increases in $[Ca^{2+}]_i$ and secretion may be due to Ca^{2+} entry or to release from internal Ca^{2+} stores or both. Perfusion with Ca^{2+}-free external solution, after VIP, decreased $[Ca^{2+}]_i$ and secretion to basal levels in a reversible fashion (Fig. 6A; $n = 22$). Application of 100 μM La^{3+} also reduced the VIP-induced $[Ca^{2+}]_i$ plateau back to baseline, with an accompanying decline in secretion (Fig. 6B; $n = 5$). Thus, the sustained increases in $[Ca^{2+}]_i$ and release evoked by VIP depend on external Ca^{2+}, suggesting that VIP promotes Ca^{2+} entry.

We found that rat chromaffin cells in culture express N-, P- and L-type whole-cell Ca^{2+} currents (M. Anderova, J.G. Barbara and K. Takeda, unpublished observations). Here, 1 μM ωCgTx, 200 nM AgaIVa and 1 μM nifedipine, respective blockers of N-, P- and L-type Ca^{2+} channels, had no effect on the long-lasting VIP-induced responses (Fig. 7A; $n = 9$). Together with the lack of effect of VIP on K^+-induced Ca^{2+} signals, this indicates that VIP does not modulate Ca^{2+} channel activity. In agreement, 10 μM VIP had no effect on whole-cell Ca^{2+} currents recorded from rat chromaffin cells using whole-cell ($n = 20$) or amphotericin B, perforated-patch ($n = 12$) techniques (not shown).

The VIP stimulation of a receptor- or second messenger-operated Ca^{2+} current [13] was tested using SK&F 96365, an antagonist of receptor-mediated Ca^{2+} entry [29]. Application of 20 μM SK&F 96365 produced a strong decrease in the VIP-induced $[Ca^{2+}]_i$ plateau ($\sim -75\%$) and an accompanying inhibition of secretion (Fig. 7B; $n = 7$).

In six of 15 cells exposed to Ca^{2+}-free external solution for 2 min, 10 μM VIP (in Ca^{2+}-free solution) produced a transient rise in $[Ca^{2+}]_i$ consistent with release of Ca^{2+} from internal stores, accompanied by a short-lived increase in secretion (Fig. 8A). In the nine other cells, VIP in the absence of Ca^{2+} was ineffective, presumably because internal stores were depleted (Fig. 8B). In all cells, when 2 mM Ca^{2+} was reintroduced in the external solution after challenge with VIP, both $[Ca^{2+}]_i$ and secretion increased

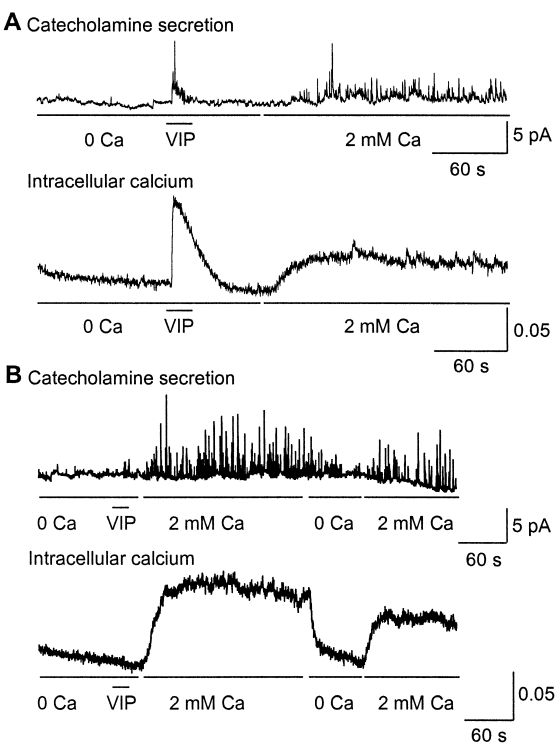

Fig. 8. Internal Ca^{2+} stores are not essential for VIP responses. Cells were exposed to Ca^{2+}-free external solution for 2 min. A 20-s application of 10 μM VIP (in Ca^{2+}-free external solution) produces either a transient release of Ca^{2+} from internal stores and a accompanying burst of secretion (A) or was without immediate effect (B). Upon reintroduction of external solution containing 2 mM Ca^{2+}, sustained increases in $[Ca^{2+}]_i$ and secretion rapidly developed in both cells.

rapidly to maintained, elevated levels (Fig. 8A,B). Such long-lived responses were not seen upon reintroduction of external Ca^{2+} in the absence of a preceding challenge with VIP (not shown), strongly suggesting that receptor-operated Ca^{2+} entry was largely predominate compared to capacitative Ca^{2+} entry. Taken together, these data confirm the dependence of sustained VIP-induced responses on external Ca^{2+}, and are consistent with VIP-inducing Ca^{2+} influx that is not uniquely dependent on an immediately prior VIP-evoked release of Ca^{2+} from internal stores. Unfortunately, our efforts to directly demonstrate a VIP-associated inward cationic current at the resting potential using both classical tight-seal whole-cell and amphotericin B, perforated patch-clamp recordings were unsuccessful (not shown).

Since VIP activates adenylate cyclase in chromaffin cells, we tried to mimic these effects of VIP using 8-Br-cAMP and forskolin. Fig. 9A shows that 50 μM 8-Br-cAMP induces transient rises in secretion but no increase in $[Ca^{2+}]_i$ ($n = 18$). In contrast, 10 μM forskolin triggered sustained increases in $[Ca^{2+}]_i$ and secretion (Fig. 9B; $n = 5$). We recently reported [37] that 8-Br-cAMP is ineffective in stimulating rises in $[Ca^{2+}]_i$ in rat chromaffin cells, and that the effects of forskolin are not likely to be mediated by the cAMP/PKA pathway, as they were mimicked by 1,9 dedeoxyforskolin, an analogue inactive in stimulating adenylate cyclase [22], and not inhibited by H-89. Rather, it was concluded that forskolin acts by block of K^+ conductance, leading to depolarization-activated Ca^{2+} entry [37]. In agreement with the inability of 8-Br-cAMP to mimic the long-lasting actions of VIP, 30-min preincubation with 200 or 400 nM H-89 had no effect on VIP-induced secretion and elevation of $[Ca^{2+}]_i$ ($n = 6$; not shown). Finally, application of 50 μM 8-Br-cGMP produced no rises in $[Ca^{2+}]_i$ ($n = 5$; not shown).

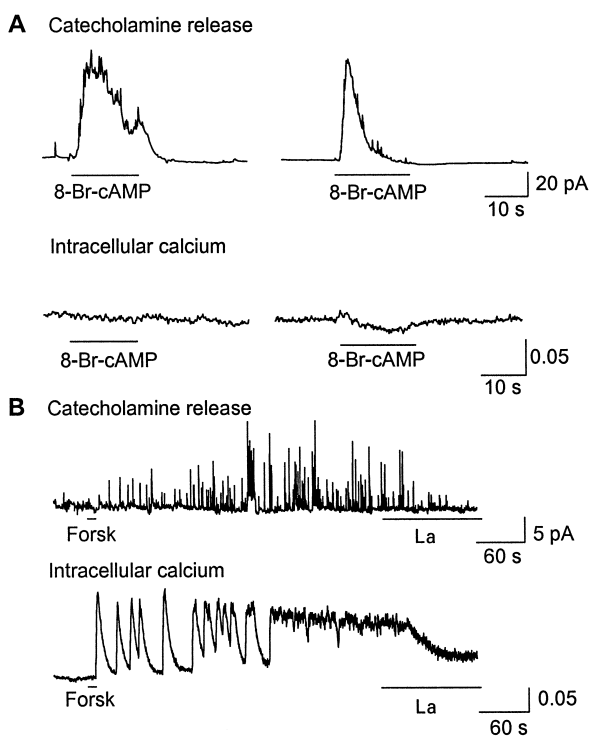

Fig. 9. Effects of 8-Br-cAMP and forskolin on $[Ca^{2+}]_i$ and secretion. (A) Two examples showing that 50 μM 8-Br-cAMP produces large transient increases in secretion without changes in $[Ca^{2+}]_i$. (B) An amount of 10 μM forskolin induced sustained increases in $[Ca^{2+}]_i$ and secretion, both blocked by bath application of 100 μm La^{3+}.

4. Discussion

4.1. Possible basis for the potentiating effect of VIP

The potentiating effects of VIP on catecholamine release from single rat chromaffin cells are consistent with previous work showing that VIP enhances secretion evoked by ACh [28] and by elevated K^+ [41]. This potentiation is apparently not correlated in a simple way to changes in $[Ca^{2+}]_i$ (Fig. 2C). For example, $[Ca^{2+}]_i$ levels and secretion to 1 μM ACh following a prior VIP application were increased by 17% and 94%, respectively, while with 60 mM K^+, no potentiation of $[Ca^{2+}]_i$ was seen and secretion was enhanced by 68%. Also, when VIP was co-applied with ACh, $[Ca^{2+}]_i$ was increased by 73% and secretion by 153%. Thus, at least with ACh, VIP enhancement of release is associated with higher levels of $[Ca^{2+}]_i$. In contrast, the effect of VIP on secretion to high K^+ seems to be independent of any further increase in $[Ca^{2+}]_i$, in accord with VIP not stimulating voltage-sensitive Ca^{2+} channels.

The potentiation by VIP of ACh-induced $[Ca^{2+}]_i$ responses appears to be mediated by muscarinic receptors. This effect of VIP was mimicked by 8-Br-cAMP, and both were blocked by H-89. It may be that muscarinic receptor function is upregulated by PKA and/or that a cAMP-dependent increase in IP_3 receptor activity occurs. The lack of effect of VIP on nicotine-mediated responses differs from a recent report in chick neurons where VIP recruited nicotinic receptors [16].

Since VIP potentiation of secretion is not entirely accounted for by changes in $[Ca^{2+}]_i$, a cAMP-dependent increase in the Ca^{2+}-sensitivity of the secretion machinery [20] might also be involved. This was proposed for the Ca^{2+}-independent potentiation of insulin release by cAMP in single pancreatic β-cells [1]. Another possibility may be related to VIP stimulating phospholipase C activity [28] and the formation of diacylglycerol, thereby stimulating protein kinase C [19]. In bovine chromaffin cells, PKC enhances exocytosis by increasing the size of the readily releasable pool of secretory granules [14]. As well, proteins like GAP-43 [3] and annexin II [9] affect neurotransmitter release [8] and are also substrates for PKC. Thus, while nothing is known about possible effects of VIP on such proteins, they might be regulated by VIP through PKC.

A striking effect of VIP co-application with ACh was the increased duration of secretion and $[Ca^{2+}]_i$ responses. Interestingly, VIP applied 130 s before ACh produces much smaller effects compared to VIP and ACh together. This may reflect temporally different VIP-activated signalling pathways. Indeed, in rat adrenal medulla, VIP acts on both the PLC–phosphoinositide and adenylate cyclase–cAMP pathways [27,28]. Thus, the PLC pathway may contribute preferentially to an immediate enhancement of secretion and $[Ca^{2+}]_i$ levels during co-application of VIP with ACh. As VIP and ACh are co-localized in presynaptic splanchnic nerve terminals [18] and can be co-released by depolarization [40], VIP-mediated enhancement of secretion evoked by ACh in chromaffin cells is of likely physiological relevance, perhaps especially so during prolonged exposure to ACh, as in stress, when nicotinic receptors may be largely desensitized.

4.2. VIP-associated increases in $[Ca^{2+}]_i$

Vasoactive intestinal polypeptide alone acts as a secretagogue, producing rises in both $[Ca^{2+}]_i$ and secretion in a remarkable, sustained manner. Such direct VIP-evoked release confirms previous work on perfused adrenal glands [15,26,28,38], suspensions of pig adrenal chromaffin cells [31] and single rat chromaffin cells [7]. In contrast, VIP-induced increases in $[Ca^{2+}]_i$ [31] or $^{45}Ca^{2+}$ uptake [27] were not reported. The dependence on external Ca^{2+} of the sustained VIP elevation in $[Ca^{2+}]_i$ together with the block by La^{3+} indicate that VIP activates Ca^{2+} influx, while omission of external Ca^{2+} had almost no effect on VIP-induced secretion in a previous study [27]. These discrepancies may reflect different methodological approaches.

Not all cells responded to VIP alone, perhaps suggesting that only certain types of chromaffin cells are VIP-sensitive. Since chromaffin cells can be separated into adrenergic and noradrenergic classes [43], and VIP and PACAP predominately stimulate the secretion of adrenaline [15], it may that adrenergic chromaffin cells are preferentially sensitive to VIP.

Vasoactive intestinal polypeptide induces a biphasic rise in $[Ca^{2+}]_i$ with the initial transient being probably due to IP_3-mediated release of Ca^{2+} from internal stores [27]. The sustained plateau involves Ca^{2+} entry, which is not mediated by voltage-dependent Ca^{2+} channels. Thus, a VIP-associated increase in cAMP leading to recruitment of L-type facilitation Ca^{2+} channels, as in calf chromaffin cells [2] challenged with dopamine, is unlikely. A cAMP-mediated inhibition of Na^+,K^+-ATPase resulting in Na^+ accumulation [32], depolarization [30] and Ca^{2+} channel activation also appears improbable.

The most likely explanation for the VIP-induced $[Ca^{2+}]_i$ plateau is activation of receptor-operated Ca^{2+} entry [13], consistent with the inhibitory effects of SK&F 96365. Note however that while SK&F 96365 was initially described as blocker of receptor-mediated Ca^{2+} entry [29], it also blocks internal store depletion-activated current mediated by putative CRAC (Ca^{2+} release-activated current) channels. VIP-associated Ca^{2+} entry appears not to involve the cAMP/PKA pathway, as H-89 was ineffective against VIP-induced responses and 8-Br-cAMP produced no $[Ca^{2+}]_i$ elevation (Fig. 9A). Speculatively, it may be that VIP causes direct gating of Ca^{2+}-permeable channels by IP_3 and/or IP_4. While VIP induces transient, external Ca^{2+}-dependent rises in $[Ca^{2+}]_i$ in rat pinealocytes perhaps via cGMP-dependent activation of diltiazem-sensitive, rod-like, cyclic nucleotide-gated cationic channels [36], here, 8-Br-cGMP was without effect. Furthermore, while the data in Fig. 8 indicate that VIP is required to produce long-lasting responses upon re-addition of Ca^{2+} independently of the filling state of internal Ca^{2+} stores, our inability to measure inward cationic current activated by VIP remains puzzling. A possible explanation is that VIP stimulates truly capacitative Ca^{2+} entry, but in this case, Ca^{2+} entering stores would have to be immediately released. Alternatively, VIP might directly activate CRAC-like channels, but the resultant whole-cell amplitude of such current is too small to be measured under our conditions.

4.3. Is cAMP involved?

Our effects of VIP in rat chromaffin cells differ from those of PACAP in elevating $[Ca^{2+}]_i$ [44] and secretion [21] in frog and porcine chromaffin cells respectively, where voltage-dependent Ca^{2+} channels were implicated. On the other hand, it was recently reported [34] that PACAP causes an external Ca^{2+}-dependent, sustained increase in secretion in single rat chromaffin cells, with associated rises in $[Ca^{2+}]_i$ being insensitive to nifedipine and ωCgTx. However in contrast, it was concluded that the cAMP/PKA cascade was involved, based on the stimulatory actions of forskolin and inhibitory effect of the PKA inhibitor, *R*p-cAMPS, on secretion [34]. Note that the PACAP receptors in chromaffin cells are of the high affinity type, having virtually no affinity for VIP [39].

Whether VIP-associated increases in cAMP levels are essential in stimulating secretion is thus unclear. Interestingly, secretagogue-mediated rises in cAMP in bovine chromaffin cells, when compared to those induced by forskolin and permeant cAMP analogues, were not strictly correlated to corresponding increases in secretion, especially when strong elevations of cAMP were produced [33] —this might argue for receptor-mediated compartmentalized effects of cAMP. In another study, direct secretagogue effects of VIP in bovine chromaffin cells were seen only after pretreatment with pertussis toxin and were not dependent on marked rises in cAMP [42]. Note that the inhibitory action of VIP on N-type Ca^{2+} currents in rat sympathetic neurons does not involve cAMP or PKA [45], unlike the cAMP-dependent activation of large conductance Ca^{2+}-sensitive K^+ channels by VIP in mouse lacrimal cells [23].

5. Conclusions

Vasoactive intestinal polypeptide potentiates ACh-induced catecholamine secretion from rat adrenal chromaffin cells, apparently via a cAMP-dependent modulation of muscarinic receptor function. Secondly, VIP also directly acts as a secretagogue, producing long-lasting secretion and maintained elevation of $[Ca^{2+}]_i$. These latter effects are likely accounted for by activation of receptor-operated Ca^{2+} entry in a cAMP-independent manner.

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