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INHIBITORY EFFECTS OF HISTAMINE AND BRADYKININ ON CALCIUM CURRENT IN SMOOTH MUSCLE CELLS ISOLATED FROM GUINEA-PIG ILEUM

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SUMMARY

1. Single smooth muscle cells were isolated from the longitudinal muscle layer of the guinea-pig ileum and within 10 h Ca^{2+}-currents (I_{Ca}) were recorded using the whole-cell patch clamp technique.

2. Histamine (10 μM) and bradykinin (BK, 1 μM) suppressed I_{Ca}; the effect had two phases: a rapid and transient suppression of I_{Ca} followed by a sustained suppression. Acetylcholine and substance P appeared to have similar effects but these were not investigated in detail.

3. The effects of histamine and BK on I_{Ca} were abolished by high intracellular concentrations of the Ca^{2+} buffer EGTA (30 mM) or 1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid (BAPTA) (5 mM) in the absence of Ca^{2+} added to the pipette solution. When $[Ca^{2+}]_i$ was strongly buffered to 125 or 190 nM by BAPTA–Ca^{2+} mixtures in the pipette the transient suppression of I_{Ca} was blocked but the sustained effect still occurred. This indicated that the transient effect was caused by a rise in $[Ca^{2+}]_i$. The sustained effect, in contrast, did not seem to be caused by a rise in $[Ca^{2+}]_i$ but did show Ca^{2+} dependence because it did not occur if $[Ca^{2+}]_i$ was abnormally low.

4. Application of caffeine (10 mM) to deplete stored Ca^{2+} or intracellular heparin (1 mM) to block the action of D-*myo*-inositol 1,4,5-trisphosphate (IP_3) to release stored Ca^{2+} prevented the transient but not the sustained suppression of I_{Ca}. Heparin also blocked the transient Ca^{2+}-activated K^+ current in response to histamine or BK. Both transient and sustained suppressions of Ca^{2+} channel activity were observed in the absence of extracellular Ca^{2+} when current was carried mostly by Na^+ ions.

5. Intracellular guanosine 5'-*O*-(3-thiotriphosphate) (GTP-γ-S; 10 or 100 μM) induced a gradual decline of I_{Ca} upon which transient decreases of current were superimposed. Histamine caused a larger than normal inhibition of I_{Ca} and no recovery occurred on wash-out. Intracellular guanosine 5'-*O*-(2-thiodiphosphate) (GDP-β-S; 1 mM) abolished the effects of histamine and BK on I_{Ca}.

6. From these results it appears that histamine and BK inhibit voltage-gated Ca^{2+} channels via two mechanisms: (a) they induce the production of IP_3 which releases Ca^{2+} from intracellular stores causing a transient Ca^{2+}-induced inactivation of the

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Ca^{2+} channels; (b) they activate a G-protein which causes a slow and long-lasting inhibition of Ca^{2+} channels via a mechanism that does not require Ca^{2+} release from stores but instead depends on $[Ca^{2+}]_i$ in such a way that it ceases to function if $[Ca^{2+}]_i$ is reduced below the resting level.

INTRODUCTION

Histamine causes contraction of smooth muscle cells in the guinea-pig ileum. There is an initial phasic contraction because D-*myo*-inositol 1,4,5-trisphosphate (IP_3) is produced (Jafferji & Michell, 1975), which releases Ca^{2+} from intracellular stores (Pacaud & Bolton, 1991), and a tonic contraction because there is membrane depolarization and an increase in spike frequency (Bolton, Clark, Kitamura & Lang, 1981), which promotes Ca^{2+} influx through voltage-gated Ca^{2+} channels. Bradykinin (BK) also contracts the ileum by a direct action (Day & Vane, 1963) and at least part of the effect is likely to occur because IP_3 is produced (Ransom, Goodman & Young, 1992).

Histamine and BK also have inhibitory effects on smooth muscle in the guinea-pig ileum. If histamine is applied during a contraction induced by high KCl there is phasic contraction but this is followed by relaxation (Bolton *et al.* 1981; Holzer & Lippe, 1984), and BK causes relaxation if the ileum is previously contracted by submaximal doses of acetylcholine (Hall & Bonta, 1973). In addition, high concentrations of histamine cause non-specific desensitization because they reduce the contractile effects of subsequent applications of histamine or acetylcholine (Cantoni & Eastman, 1946; Horio, Shima, Ueda & Ishida, 1990; Himpens, Droogmans & Casteels, 1991).

This paper examines the novel observation that histamine and BK inhibit voltage-gated Ca^{2+} channels in smooth muscle cells isolated from the guinea-pig ileum. Part of the inhibition was expected to result from the production of IP_3 because it has been shown previously that IP_3 caused a transient inhibition of Ca^{2+} current (I_{Ca}) by raising the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in smooth muscle cells from the rabbit jejunum (Komori & Bolton, 1991*a*). However, additional mechanisms for Ca^{2+} channel inhibition may also exist. In smooth muscle cells from the guinea-pig vas deferens, noradrenaline suppressed I_{Ca} mostly by a Ca^{2+}-independent mechanism that seemed to require a GTP-binding protein (Imaizumi, Takeda, Muraki & Watanabe, 1991). Such a Ca^{2+}-independent G-protein hypothesis is reminiscent of that often proposed for the action of neurotransmitters on neuronal Ca^{2+} channels (reviewed by Anwyl, 1991). However, in these studies investigators have often employed high concentrations of EGTA or 1,2-bis(*O*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) (Ca^{2+} buffers) in the recording pipette which may diminish the role of Ca^{2+}-dependent pathways operating in parallel (Beech, Bernheim, Mathie & Hille, 1991). This is not surprising because Ca^{2+} chelators can strongly reduce normal $[Ca^{2+}]_i$ below the level required for many cellular enzymes. Therefore, one aim of the present study was to investigate the effects of Ca^{2+} chelators on Ca^{2+} channel modulation in the guinea-pig ileum. The results seem to suggest that histamine and BK acted similarly on Ca^{2+} channels and that they both acted via two Ca^{2+}-dependent mechanisms.

METHODS

Preparation of cells

Guinea-pigs (300–500 g, male or female) were killed by stunning and exsanguination and the longitudinal muscle was pealed from a region of the ileum more than 10 cm above the ileocaecal valve. Three sections of muscle (each about 3 mm long) were preincubated in 2 ml of the dispersion solution (see 'Bath solutions') at 37 °C for 10 min and then this solution was replaced by 1 ml of the enzyme mixture and the incubation continued for 17–22 min. The enzyme mixture was 0·2–0·4 mg ml^{-1} collagenase (Type XI) and 0·2–0·4 mg ml^{-1} protease (Type XXV) in the dispersion solution. The enzyme mixture was then removed and fresh dispersion solution added at room temperature. The tissue was agitated mechanically until a suspension of isolated cells was generated. Cells were stored in dispersion solution (0·2 mM calcium added) at 4 °C and recorded from within 10 h.

Recordings

Whole-cell voltage clamp of single cells (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) was obtained by using an Axopatch-1C amplifier (Axon Instruments Inc., CA, USA) and borosilicate glass recording pipettes of 1–2·5 MΩ (Clark Electromedical Instruments, UK). Capacity currents were partially cancelled and signals were filtered at 1 kHz (−3 dB) by circuitry in the Axopatch-1C. The series resistance was between 3 and 10 MΩ. Command voltages were generated and signals captured on-line using a TL-1 DMA interface and the pClamp software (Axon Instruments Inc.) run on an IBM-compatible 286 computer. Signals were digitized at 2 kHz (40 ms test steps), 4 kHz (20 ms steps) or 8 kHz (10 ms steps). Figures were drawn using a Hewlett-Packard plotter. The analog recording was on FM-tape (Racal) and was displayed using a Gould 2400S chart recorder. Command voltages are shown after correction for junction potentials (see below for values) except for the nystatin-mode experiment shown in Fig. 1*A*. Fresh cells were placed in a 100 μl chamber about 10 min prior to each recording. Once the recording was started solution flowed continuously through the chamber at about 2 ml min^{-1}. Test solutions were applied by switching manually to another reservoir connected to the inflow. Solution exchange was about 90% complete 30 s after switching. Time is described for each experiment as time after breaking the cell-attached patch to gain access to the whole-cell (t_{wc}). For the nystatin-mode experiment, time is that after forming a seal. All experiments were performed at room temperature (21–26 °C). Mean values are given ± S.E.M.

Bath solutions

The '2 mM Ca^{2+}' solution contained (mM): NaCl, 150; KCl, 2·5; $CaCl_2$, 2; $MgCl_2$, 1; Hepes, 10; glucose, 8. The '0 Ca^{2+}' solution contained (mM): NaCl, 150; KCl, 2·5; EGTA, 1; Hepes, 10; glucose, 8. 'Dispersion' solution contained (mM): NaCl, 126; KCl, 6; glucose, 14; Hepes, 10. All of these solutions were titrated to pH 7·4 with NaOH.

Pipette solutions

Composition of pipette solutions (mM) was as follows. 'Cs^+–low-EGTA': CsCl, 150; $MgCl_2$, 5; Hepes, 5; EGTA, 0·1 or 0·3. 'Cs^+–high-EGTA': CsCl, 110; $MgCl_2$, 5; Hepes, 5; EGTA, 30. 'Cs^+–5 mM BAPTA–0 Ca^{2+}': CsCl, 130; $MgCl_2$, 5; Hepes, 10; BAPTA, 5. 'Cs^+–20 mM BAPTA–10 or 12 mM Ca^{2+}': CsCl, 85; $MgCl_2$, 5; $CaCl_2$, 10 or 12; Hepes, 10; BAPTA, 20. 'K^+–low-EGTA': KCl, 150; $MgCl_2$, 5; Hepes, 5; EGTA, 0·1. Solutions were titrated to pH 7·4 with CsOH or KOH. ATP (3 mM) and GTP (0·1 mM) (from frozen stock solutions at pH 7·4) were added each day to fresh aliquots of pipette solution. Free Ca^{2+} concentrations in the BAPTA–Ca^{2+} mixtures were determined using stability constants from Tsien (1980) and took into consideration Ca^{2+}, Mg^+ and H^+ binding to BAPTA and ATP (calculations were performed by the program Eqcal). Nystatin was prepared as a 50 mg ml^{-1} stock solution in 100% dimethylsulphoxide (DMSO) on the day of the experiment and diluted into the Cs^+–low-EGTA solution to a final concentration of 0·2 mg ml^{-1} (0·4% v/v DMSO). The osmolality of all solutions was measured (300–330 mosmol kg^{-1}) and the water was Milli-Q reagent grade. Junction potentials were measured using a 3 M KCl reference electrode; against the 2 mM Ca^{2+} bath solution they were (pipette negative): Cs^+–low-EGTA, −3 mV; Cs^+–high-EGTA, −7 mV; Cs^+ 5 mM BAPTA–0 Ca^{2+}, −5 mV; Cs^+–20 mM BAPTA–10 mM Ca^{2+}, −6 mV.

Chemicals

Bradykinin and substance P were obtained from Novabiochem (UK), CsCl (99·9% pure) from Aldrich (UK) and NaCl, KCl and glucose from BDH (UK). All other substances were from Sigma (UK). Abbreviations: BK, bradykinin; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; EGTA, ethylene glycol-bis (*β*-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; ATP, disodium adenosine 5'-triphosphate; GTP, sodium guanosine 5'-triphosphate; GTP-*γ*-S, lithium guanosine 5'-O-(3-thiotriphosphate); GDP-*β*-S, lithium guanosine 5'-O-(2-thiodiphosphate).

RESULTS

The nystatin-mode of whole-cell recording permits the study of agonist effects on ionic channels while avoiding obvious interference with second messenger systems (Horn & Marty, 1988). Figure 1*A* shows that bath-applied histamine caused a biphasic suppression of I_{Ca} with no effect on the holding current at −58 mV. Initially, I_{Ca} decreased rapidly by 46% and then there was some recovery before a second phase of inhibition developed and a final 50% loss of I_{Ca} occurred. This second sustained effect continued until histamine was washed from the bath.

Figure 1*B* shows the effect of histamine when using conventional whole-cell recording (Hamill *et al.* 1981). The effect was qualitatively similar to that observed in the nystatin mode: there was transient and sustained inhibition of I_{Ca} and recovery on wash-out. Therefore, because the nystatin mode did not allow the introduction of test substances into cells and the high series resistance prevented reliable recording of I_{Ca}, all subsequent recordings used the conventional whole-cell configuration. In twenty-seven whole-cell recordings with the Cs^+–low-EGTA pipette solution 10 μM histamine inhibited I_{Ca} by 77±2% (transient) and 64±3% (sustained) (t_{WC} = 5–10 min).

Schneider, Hopp & Isenberg (1991) showed that in guinea-pig urinary bladder smooth muscle cells 20 ms steps to 0 mV every 1·2 s caused small $[Ca^{2+}]_i$ transients that did not decay completely before the next step. In the present experiments 20 ms steps were usually applied every 4 s, but to investigate if $[Ca^{2+}]_i$ accumulation might have affected the histamine response 20 ms steps were applied every 30 s in three experiments. Histamine (10 μM) caused a sustained 60±8% reduction of I_{Ca}, suggesting the sustained response did not depend on the frequency at which I_{Ca} was elicited. In the presence of pyrilamine (10 μM), an H_1-receptor antagonist, histamine (10 μM) had no effect on I_{Ca} (not shown). Histamine had no obvious effect on the I_{Ca}–voltage relationship apart from to reduce the overall amplitude (not shown).

Bradykinin (BK) had an effect on I_{Ca} similar to that of histamine (Fig. 2). BK (100 nM) caused a biphasic suppression of I_{Ca} where transient inhibition was 72% and sustained 80%. I_{Ca} did not recover on wash-out and 1 μM BK had no further effect. In nine cells tested with the Cs^+–low-EGTA pipette solution 1 μM BK inhibited I_{Ca} by 73±3% (transient) and 68±4% (sustained) (t_{WC} = 4–7 min). In two nystatin-mode recordings BK had a similar effect (not shown). Current–voltage relationships in control conditions and with 1 μM BK in the bath (Fig. 2*C*) showed that, as for the action of histamine, the suppression of I_{Ca} was not obviously voltage dependent. In addition, it appeared that other Ca^{2+}-dependent conductances were not contaminating the recording of I_{Ca}; this finding was supported by the observation that the response to BK was unaffected by inclusion in the bath of 10 mM

tetraethylammonium chloride (an effective blocker of large conductance Ca^{2+}-activated K^+ channels).

Acetylcholine (1 or 10 μM) also caused transient and sustained inhibition of I_{Ca} and there was recovery on wash-out (not shown). Substance P (50 or 100 nM) had a

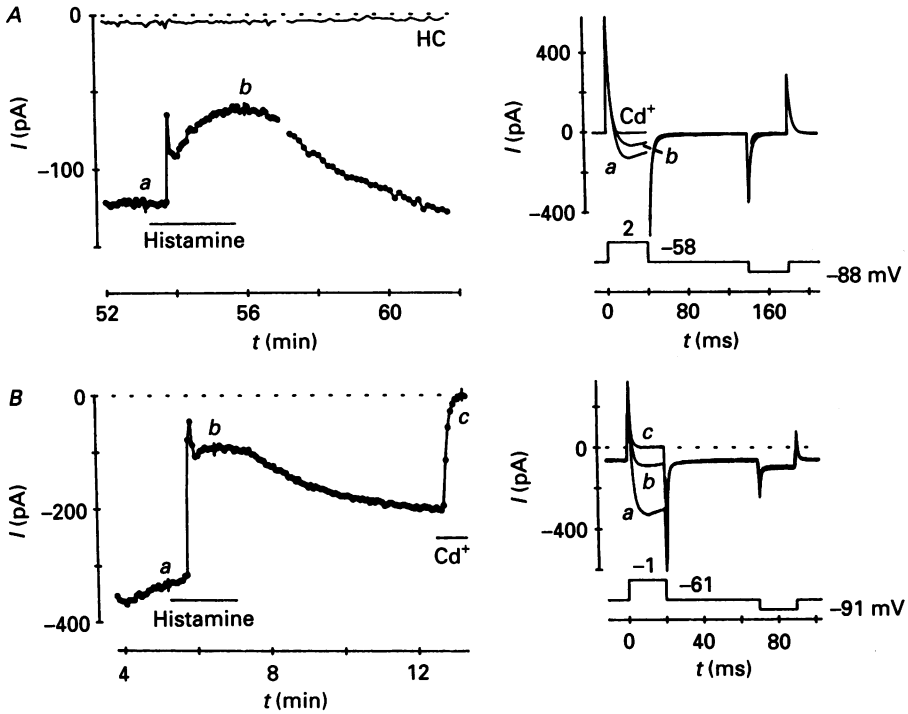

Fig. 1. Histamine-induced suppression of Ca^{2+} current (I_{Ca}). *A*, nystatin-mode recording with the Cs^+–low-EGTA pipette (no ATP or GTP) and 2 mM Ca^{2+} bath solutions. The filled circles are peak I_{Ca} measured every 4 s, and every 8 s after the break in sampling. The record below zero current is the holding current (HC) at −58 mV. Histamine (10 μM) was bath applied. Actual currents without capacity current cancellation are shown on the right with the labels *a* and *b* indicating that the currents were elicited at the times marked *a* and *b* in the plot on the left. *B*, conventional whole-cell recording with the Cs^+–low-EGTA pipette and 2 mM Ca^{2+} bath solutions. The filled circles are the peak inward current without leak subtraction at −1 mV. Histamine (10 μM) was bath applied. Cd^+ (100 μM) in the bath abolished I_{Ca}, giving zero current at −1 mV. Actual currents are shown on the right. All subsequent plots of I_{Ca} amplitudes used the current level in 100 μM Cd^+ at the test potential (determined in each experiment) as zero I_{Ca}.

similar effect except that there appeared to be desensitization because I_{Ca} gradually returned to its control amplitude while substance P was still present (not shown).

Effect of high concentrations of intracellular Ca^{2+} buffer

When the concentration of Ca^{2+} buffer in the recording pipette was fairly low (0·1–0·3 mM EGTA) the effects of histamine and BK appeared similar to those observed with nystatin-mode recording. However, if there was 30 mM EGTA in the pipette neither histamine nor BK had any effect on I_{Ca} (Fig. 3).

For these experiments the test (Cs^+–high-EGTA) and control (Cs^+–low-EGTA) pipette solutions were compared alternately (t_{WC} = 5–10 min). In the five control cells, I_{Ca} was 168±39 pA (4±1 pA pF^{-1}) and 10 μM histamine caused a 67±4% sustained inhibition. In the five test cells, I_{Ca} was 547±74 pA (13±2 pA pF^{-1}) and

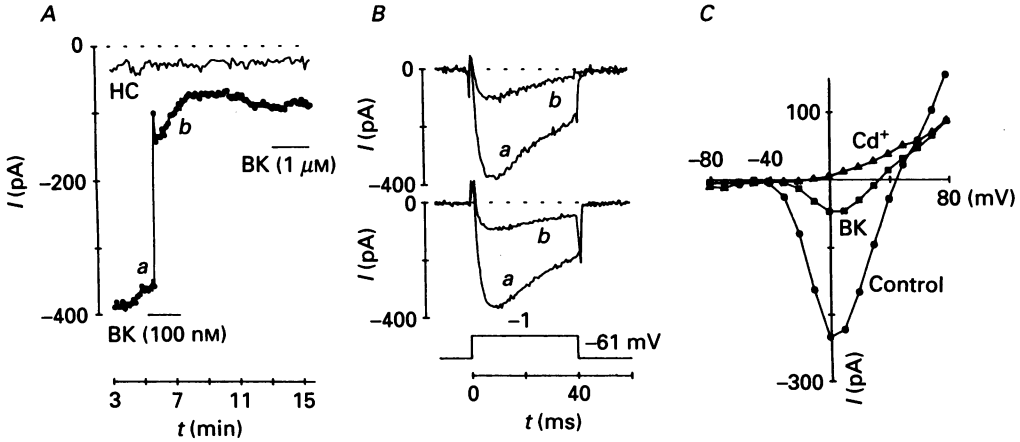

Fig. 2. Bradykinin (BK) suppressed I_{Ca}. *A*, plot of peak I_{Ca} (●) and holding current (HC) at −61 mV measured every 8 s. This experiment and those in all subsequent figures used conventional whole-cell recording. The solutions were Cs^+–low-EGTA (pipette) and 2 mM Ca^{2+} (bath) and the voltage protocol was the same as that in Fig. 1*A*. BK was bath applied. *B*, currents during the step to −1 mV immediately before (*a*) and during (*b*) the response to 100 nM BK with non-Ca^{2+} current (leak) subtracted by two methods. For the linear subtraction (upper), current during the step to −91 mV in the same sweep was multiplied by 2 and added to current during the step to −1 mV. For the Cd^+ subtraction, current elicited in 100 μM Cd^+ (at the end of the experiment) was subtracted. *C*, current–voltage relationships in control conditions (●) and with 1 μM BK (■) and then 100 μM Cd^+ (▲) in the bath. Leak current was not subtracted.

10 μM histamine had no effect on I_{Ca} in any of these that was distinguishable from run-down. BK (1 μM) was applied after histamine in four of these cells and there was also no effect. If 5 mM BAPTA was used instead of 30 mM EGTA the effect of 10 μM histamine was again blocked; I_{Ca} was inhibited by only 5±2% (n = 4).

These results confirm observations made in smooth muscle cells from the rabbit portal vein (Ohya, Kitamura & Kuriyama, 1988) and guinea-pig urinary bladder (Schneider *et al.* 1991) which suggest that Ca^{2+} channels are normally substantially inhibited by resting $[Ca^{2+}]_i$. They also suggest that the responses to histamine and BK did not occur if $[Ca^{2+}]_i$ was buffered strongly at low levels. Therefore, it could be that the agonists normally acted by changing $[Ca^{2+}]_i$ and/or that they acted by a mechanism that simply needed normal $[Ca^{2+}]_i$.

Effects of caffeine and heparin

Ca^{2+} measurements using the dye indo-1 have shown that carbachol caused both a transient and sustained rise in $[Ca^{2+}]_i$ in guinea-pig ileum smooth muscle cells which seemed to occur because IP_3 released Ca^{2+} from intracellular stores (Pacaud &

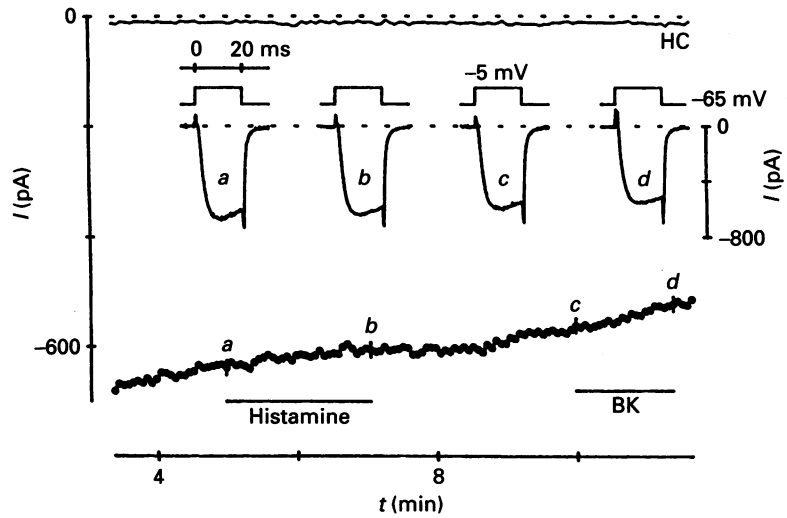

Fig. 3. Effect of high intracellular EGTA (30 mM). Plot of peak I_{Ca} (●) and holding current at −65 mV (HC) measured every 4 s. Linear leak-subtracted currents are inset, with the labels *a–d* referring to the plot of peak I_{Ca} against time and indicating when the currents were elicited during the experiment. In all subsequent figures the lower case letters, *a*, *b*, *c* etc., are used for the same purpose. Histamine (10 μM) and BK (1 μM) were bath applied. Cs^+–high-EGTA (pipette) and 2 mM Ca^{2+} (bath) solutions were used.

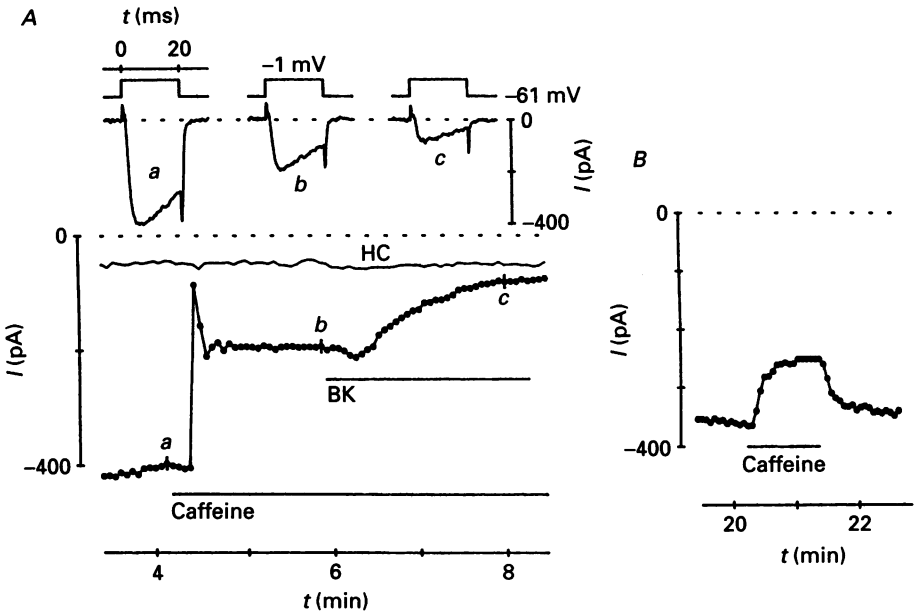

Fig. 4. Effect of caffeine pretreatment. *A*, plot of peak I_{Ca} (●) and holding current at −61 mV (HC) measured every 4 s. Linear leak-subtracted currents were shown above the plot. Caffeine (10 mM) and BK (1 μM) were bath applied. The solutions were Cs^+–low-EGTA (pipette) and 2 mM Ca^{2+} (bath). *B*, peak I_{Ca} measured every 4 s. Caffeine (10 mM) was bath applied and the solutions were Cs^+–high-EGTA (pipette) and 2 mM Ca^{2+} (bath).

Bolton, 1991). It might be that histamine and BK caused similar increases in $[Ca^{2+}]_i$ which inhibited the Ca^{2+} channels via a Ca^{2+}-induced inactivation mechanism (reviewed by Eckert & Chad, 1988). Therefore, the dependence of the histamine and BK effects on Ca^{2+} stores and on the action of IP_3 at its receptor on these stores was investigated.

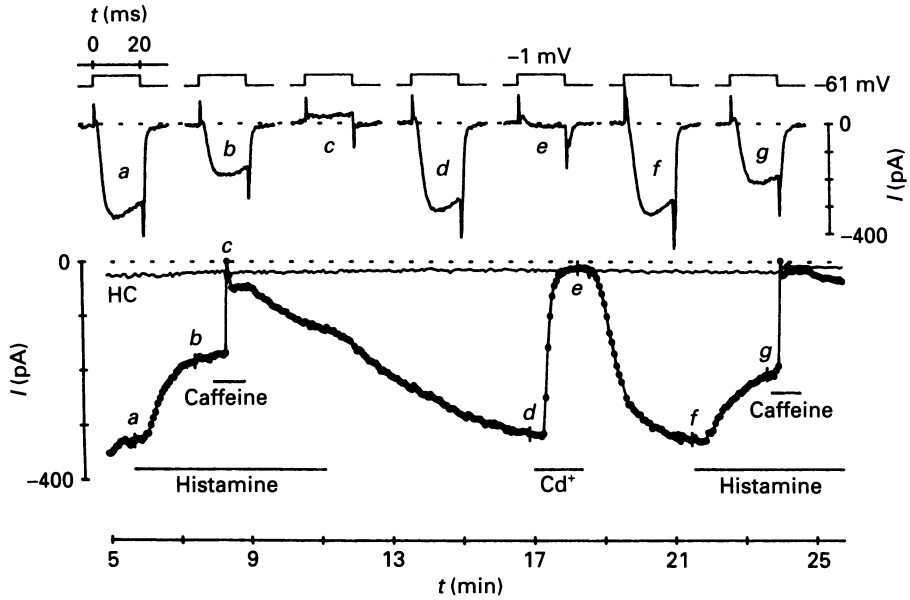

Fig. 5. Effect of intracellular heparin. The plot is of peak I_{Ca} (●) and holding current at −61 mV (HC) measured every 4 s. Linear leak-subtracted currents are shown above the plot. The solutions were Cs^+–low-EGTA (pipette) with 5 mg ml^{-1} heparin added and 2 mM Ca^{2+} (bath). Histamine (10 μM), caffeine (10 mM) and Cd^+ (100 μM) were bath applied. Cd^+ did not reduce the inward current at −1 mV to zero in this cell, perhaps suggesting the presence of a small Cd^+-resistant I_{Ca}.

Caffeine releases Ca^{2+} from intracellular stores (Komori & Bolton, 1991*b*). Figure 4*A* shows that caffeine itself caused a transient and sustained inhibition of I_{Ca} and then BK (plus caffeine) caused a sustained 60% inhibition of the remaining I_{Ca}. The usual transient effect of BK was absent in all five cells investigated and the mean sustained inhibition of I_{Ca} was 44 ± 6% ($n = 5$). Caffeine treatment also abolished the transient effect of histamine on I_{Ca} but left a sustained 29 ± 1% suppression ($n = 3$). Figure 4*B* shows inhibition of I_{Ca} by 10 mM caffeine when the pipette solution contained 30 mM EGTA. Histamine and BK had been applied to this cell and neither had affected I_{Ca}. Therefore, at least part of the sustained suppression seen with caffeine (refer also to Zholos, Baidan & Shuba, 1991) occurred by a mechanism distinct from that activated by histamine or BK.

Heparin blocks IP_3-induced Ca^{2+} release (Ghosh, Eis, Mullaney, Ebert & Gill, 1988; Komori & Bolton, 1991*b*). Figure 5 shows that histamine caused a 50% slowly developing inhibition of I_{Ca} when there was 5 mg ml^{-1} heparin in the recording pipette and that the usual transient effect was absent ($t_{WC} = 6$ min). When caffeine

(10 mM) was applied there was a large inhibition of I_{Ca}, which suggested that caffeine had released Ca^{2+} from stores that had not been affected by histamine. Second applications of histamine and caffeine had similar effects on I_{Ca} (t_{WC} = 22 min).

The response to histamine (10 μM) was investigated in six cells with 5 mg ml^{-1} heparin in the pipette; the mean inhibition of I_{Ca} was 33±3% (t_{WC} = 5–10 min) and there was no transient effect in any of the cells. In the alternating controls (no heparin) the transient inhibition of I_{Ca} was 76±8% and the sustained 59±8% (n = 5). BK (1 μM) was tested in six heparin-loaded cells (t_{WC} = 5–15 min) and I_{Ca} was inhibited by 27±6%, and again the transient effect was always abolished. The four control recordings gave transient inhibition of I_{Ca} of 72±6% and sustained of 69±8%.

Therefore, a hypothesis that both transient and sustained suppression of I_{Ca} occurred because IP_3 induced Ca^{2+} release appeared only partially correct. It seemed true for the transient effect but the sustained effect must have occurred via some other mechanism that was still effective when IP_3 could not release Ca^{2+} from intracellular stores.

Dependence on extracellular calcium

Sustained agonist-induced increases in $[Ca^{2+}]_i$ can arise as a result of Ca^{2+}-influx (Pacaud & Bolton, 1991). To examine if sustained suppression of I_{Ca} occurred because there was Ca^{2+} influx and raised $[Ca^{2+}]_i$ the extracellular Ca^{2+} concentration was reduced to less than 0·1 μM. Under this condition monovalent cation flow through Ca^{2+} channels was dominant and the voltage-dependent inward current increased from 170±16 pA (I_{Ca}) to 1302±171 pA ($I_{Na(Ca)}$) (n = 18). $I_{Na(Ca)}$ was abolished by 1 μM nicardipine, a dihydropyridine Ca^{2+} antagonist (not shown).

Figure 6 shows the effect of 10 μM histamine on $I_{Na(Ca)}$. The response was similar to that observed in the presence of 2 mM extracellular Ca^{2+}: there was transient inhibition followed by sustained inhibition and recovery on wash-out. However, the second application of histamine gave a different response: $I_{Na(Ca)}$ was inhibited slowly and there was no transient effect. This second effect was similar to that seen after caffeine or heparin treatment. The effect of 10 μM histamine was investigated in eleven cells (each 2–3 min after removing extracellular Ca^{2+}) and the mean transient inhibition of $I_{Na(Ca)}$ was 65±4% and the sustained 44±4%. BK (1 μM) caused a mean transient inhibition of 64±9% (n = 7) and a sustained of 41±5% (n = 8) (in one cell a distinct transient effect was not observed).

Therefore, neither transient nor sustained inhibition depended on extracellular Ca^{2+} in the short-term. Although the responses were smaller than those in the presence of extracellular Ca^{2+}, and may have become less if the time in the absence of extracellular Ca^{2+} had been increased (this was not investigated), it is likely that such effects may only have reflected a dependence of $[Ca^{2+}]_i$ on extracellular Ca^{2+}.

Ca^{2+}-dependent K^+ currents

Agonist effects on Ca^{2+}-dependent K^+ currents were examined to investigate the temporal changes in $[Ca^{2+}]_i$ (Komori & Bolton, 1991*b*). These recordings also tested if 5 mg ml^{-1} heparin was sufficient to completely block IP_3-induced Ca^{2+} release.

Figure 7*A* shows the effects of histamine and BK on K^+ currents measured every 4 s. Both agonists increased K^+ current transiently. The effect was brief (< 8 s) at −61 and −41 mV and more prolonged at +59 mV (lasting about 24 s) where large conductance Ca^{2+}-dependent K^+ channels were more sensitive to $[Ca^{2+}]_i$ (reviewed by

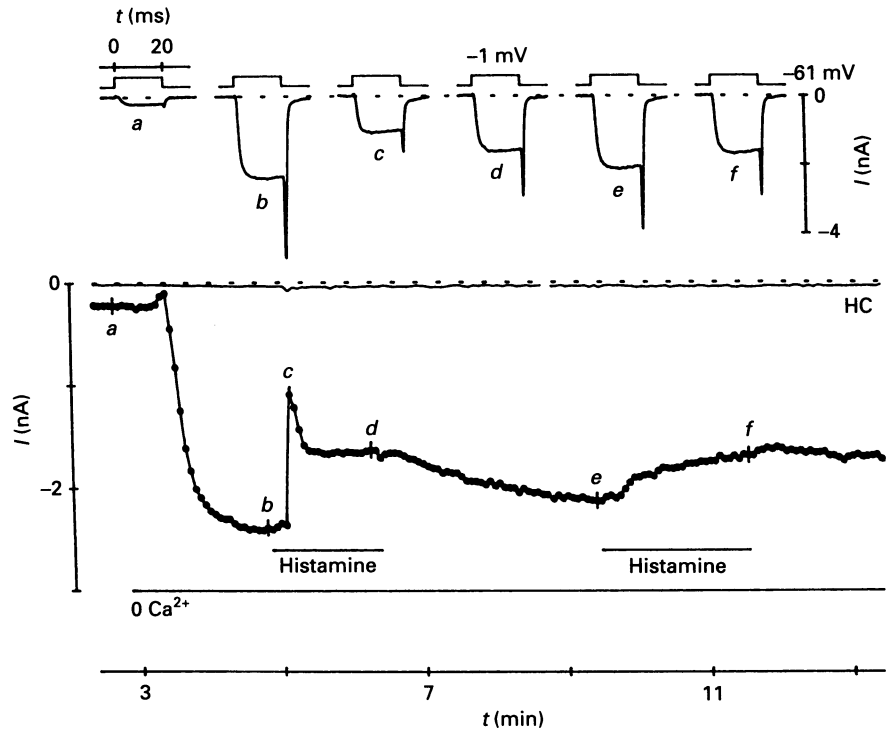

Fig. 6. Action of histamine in the absence of extracellular Ca^{2+}. Lower panel, plot of peak inward current (●) measured every 4 s. Linear leak-subtracted currents are shown above the plot. The bath solution was changed from 2 to 0 mM Ca^{2+} at $t_{WC} = 3$ min, 10 μM histamine was bath applied twice (as marked) and the pipette solution was Cs^+–low-EGTA. In other experiments, series resistance was compensated by up to 70% and similar responses were observed. In two cells in 0 Ca^{2+} bath solution the peak of the $I_{Na(Ca)}$–voltage relationship was at −18 mV and the mid-point of the steady-state inactivation curve at −43 mV; in the 2 mM Ca^{2+} bath solution these values were +5 mV and −35 mV respectively.

Bolton & Beech, 1992). There was no sustained current increase although occasional small transients were observed at +59 mV while histamine was still present (see below).

Figure 7*C* shows that the effect of BK on K^+ current was absent when 5 mg ml^{-1} heparin was in the recording pipette. Subsequent application of 10 mM caffeine did cause a large K^+ current, suggesting that intracellular Ca^{2+} stores did contain Ca^{2+}. In ten out of eleven control recordings 1 μM BK caused a large increase in K^+ current, but in all six cells with 5 mg ml^{-1} heparin in the pipette BK had no effect. A similar result was observed for histamine: in six out of eight controls 10 μM histamine

strongly increased the K^+ current, but with heparin in the pipette three cells showed no response and in the fourth outward current at +59 mV increased by < 1 nA and a second application of histamine gave no response.

Therefore, the Ca^{2+} dependent K^+ current recordings indicated that histamine and BK elicited large transient rises in $[Ca^{2+}]_i$. A sustained increase in $[Ca^{2+}]_i$ was not

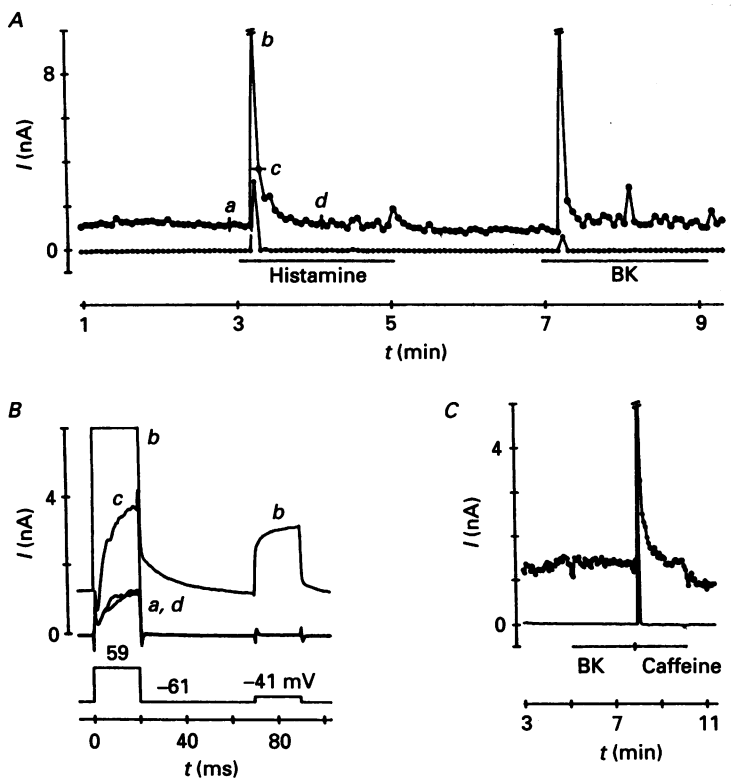

Fig. 7. $[Ca^{2+}]_i$ indicated by Ca^{2+}-dependent K^+ current. *A*, plot of K^+ current amplitude at +59 mV (upper plot, larger filled circles) and at −41 mV (lower plot) measured every 4 s. The solutions were 2 mM Ca^{2+} (bath) with 1 μM nicardipine and 1 mM 3,4-diaminopyridine added, and K^+–low-EGTA (pipette). Histamine (10 μM) and BK (1 μM) were bath applied. Peak responses at +59 mV exceeded 10 nA and were not recorded. (Similar effects were observed in the absence of nicardipine and 3,4-diaminopyridine.) *B*, actual currents for the experiment described in *A*. *C*, K^+ current at +59 mV (upper) and at −41 mV (lower) when 5 mg ml^{-1} heparin had been included in the pipette solution (K^+–low-EGTA). BK (1 μM) and caffeine (10 mM) were bath applied.

indicated and heparin (5 mg ml^{-1}) was found to be sufficient to prevent the $[Ca^{2+}]_i$ rise.

The role of $[Ca^{2+}]_i$ in the sustained suppression of I_{Ca}

High intracellular EGTA or BAPTA blocked the effects or histamine or BK (Fig. 3). The block of sustained suppression could have resulted from a decrease in $[Ca^{2+}]_i$ or from another action of the EGTA/BAPTA molecule that was independent of its

Ca^{2+} chelating property (Beech *et al.* 1991). These possibilities were addressed by raising $[Ca^{2+}]_i$ in the presence of a high concentration of BAPTA.

Figure 8*A* shows the effect of 10 μM histamine when the pipette contained a mixture of 20 mM BAPTA and 12 mM Ca^{2+}. There was a slowly developing 35%

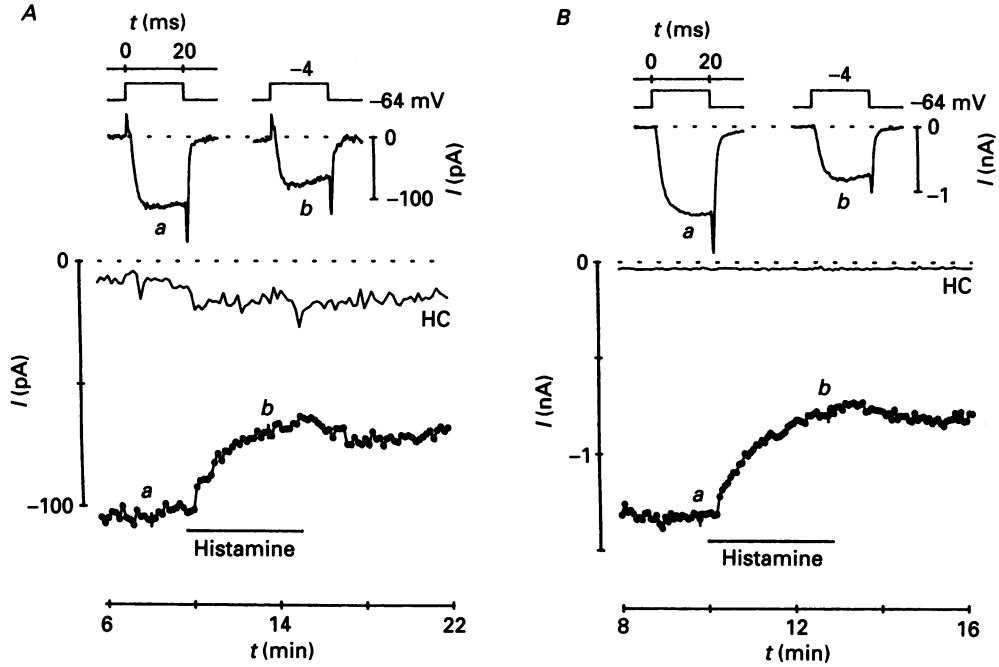

Fig. 8. Histamine responses when $[Ca^{2+}]_i$ was strongly buffered near the resting level. *A*, plot of peak I_{Ca} (●) and holding current at −64 mV (HC) measured every 10 s. Linear leak-subtracted currents are shown above the plot. Histamine (10 μM) was bath applied and the solutions were 2 mM Ca^{2+} (bath) and Cs^+–20 mM BAPTA–12 mM Ca^{2+} (pipette; calculated free Ca^{2+}, 190 nM). *B*, peak inward current $I_{Na(Ca)}$ (●) and holding current at −64 mV (HC) measured every 4 s. Histamine (10 μM) was bath applied and linear leak-subtracted currents are shown above the plot. The solutions were 0 Ca^{2+} (bath; 0 to 2 mM Ca^{2+} occurred at t_{WC} = 2·5 min) and Cs^+–20 mM BAPTA–10 mM Ca^{2+} (pipette; calculated free Ca^{2+}, 125 nM).

inhibition of I_{Ca} and some recovery occurred on wash-out. The transient effect of histamine was absent which suggested that Ca^{2+} release from stores had either been prevented or that the $[Ca^{2+}]_i$ increase had been effectively chelated by BAPTA. Figure 8*B* shows a similar experiment but in the absence of extracellular Ca^{2+}. The pipette solution contained 20 mM BAPTA and 10 mM Ca^{2+} and histamine inhibited $I_{Na(Ca)}$ by 44%. Again the response developed slowly and there was no transient effect. If the pipette solution contained 20 mM BAPTA with 10 mM Ca^{2+} or 12 mM Ca^{2+} and the 2 mM Ca^{2+} bath solution was used I_{Ca} was inhibited by 30±4% (t_{WC} = 10 min; n = 6). In two cells, 1 μM BK inhibited I_{Ca} by 28%. In six cells tested in the absence of extracellular Ca^{2+} the mean inhibition of I_{Ca} caused by 10 μM histamine was 32±4% (t_{WC} = 10 min). In all of these experiments the transient effect was absent.

From these experiments it appears that sustained suppression could occur in the presence of intracellular BAPTA provided that some $[Ca^{2+}]_i$ was also present. The experiments also support the view that sustained suppression occurred without a change in $[Ca^{2+}]_i$.

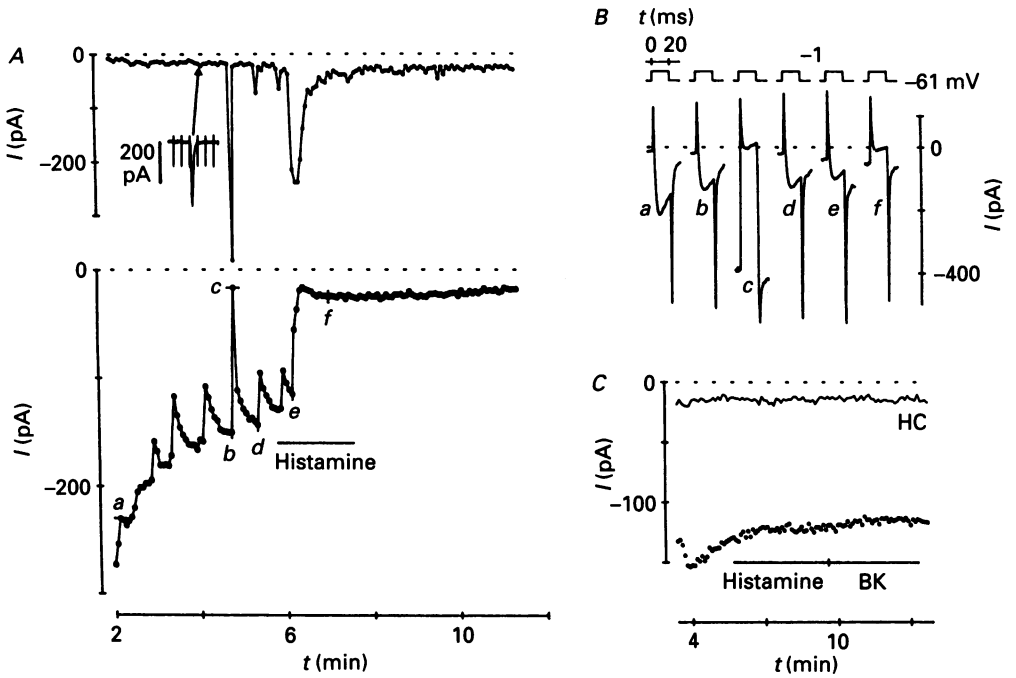

Fig. 9. Effects of intracellular stable guanine nucleotides. *A*, plots of peak I_{Ca} (lower) and holding current at −61 mV (upper) measured every 4 s. The inset in the upper plot is an analog recording showing current missed when measuring every 4 s (the vertical tick marks on the record indicate when the test steps were applied). The solutions were 2 mM Ca^{2+} (bath) and Cs^+–low-EGTA (pipette) with 10 μM GTP-γ-S added (no GTP). Histamine (10 μM) was bath applied. *B*, actual (not leak-subtracted) currents for the experiment described in *A*. *C*, plot of peak I_{Ca} (dots) and holding current at −61 mV (HC) measured every 4 s. The solutions were 2 mM Ca^{2+} (bath) and Cs^+–low-EGTA (pipette) with 1 mM GDP-β-S added. Histamine (10 μM) and BK (1 μM) were bath applied.

GTP-binding proteins

Histamine (H_1) and BK receptors are considered to be in the G-protein-coupled receptor class (Birnbaumer, Abramowitz & Brown, 1990). To test if this was true for coupling to Ca^{2+} channels in the guinea-pig ileum the effects of stable guanine nucleotides on the histamine and BK responses were investigated.

Figure 9*A* describes a recording where there was 10 μM GTP-γ-S in the pipette instead of the usual 100 μM GTP. Before histamine was applied there was a gradual decline in the amplitude of I_{Ca} and both the holding current at −61 mV and I_{Ca} began to change transiently and rhythmically. The changes in the holding current closely resembled those observed in response to carbachol (Komori, Kawai, Takewaki

& Ohashi, 1992) and may reflect transient elevations of $[Ca^{2+}]_i$ which open cationic channels sensitized to $[Ca^{2+}]_i$ by the action of an activated G-protein (Inoue & Isenberg, 1990*a*, *b*; T. B. Bolton, S. Komori, & P. Pacaud, personal communication). The transient decreases in I_{Ca} may also have resulted from elevations of $[Ca^{2+}]_i$ but they differed from the transient inward currents at -61 mV because their duration was longer.

When histamine was bath applied and GTP-γ-S was in the pipette, I_{Ca} was strongly inhibited and inward current occurred at the holding potential (Fig. 9*A*). The inward current declined back to the baseline while histamine was still present but I_{Ca} remained suppressed (80% inhibition), even after wash-out. In the four cells loaded with 10 μM GTP-γ-S histamine (10 μM) caused a sustained inhibition of I_{Ca} of $71 \pm 8\%$ ($t_{WC} = 5$–6·5 min). In three cells loaded with 100 μM GTP-γ-S I_{Ca} was inhibited by $92 \pm 3\%$. The I_{Ca} did not recover on wash-out of histamine in any of the cells. This maintained effect seems unlikely to have been mediated by a sustained elevation of $[Ca^{2+}]_i$ but rather by an irreversible activation of the caffeine- and heparin-resistant inhibitory mechanism. In the alternating control cells (no GTP-γ-S or GTP in the pipette), histamine (10 μM) caused a sustained inhibition of I_{Ca} of $61 \pm 8\%$ ($n = 5$), suggesting that GTP-γ-S may have potentiated the histamine effect.

When the pipette solution contained 1 mM GDP-β-S I_{Ca} appeared normal but neither histamine nor BK had any effect (Fig. 9*C*). Four cells were tested and none responded to histamine (10 μM); 1 μM BK was tested in two of these and also had no effect. In three alternating control cells, sustained inhibition caused by 10 μM histamine was $56 \pm 14\%$ (one of these is shown in Fig. 1*B*). Because the GDP-β-S also contained Li^+ the effect of 3 mM LiCl without GDP-β-S was tested but neither transient nor sustained effects of histamine were blocked.

These experiments suggest that a G-protein was necessary for the coupling of histamine (H_1) and BK receptors to Ca^{2+} channels in the guinea-pig ileum.

DISCUSSION

This study investigated the mechanism of histamine- or BK-induced inhibition of voltage-gated Ca^{2+} channels in the longitudinal muscle layer of the guinea-pig ileum. A working hypothesis has been developed where the activation of either histamine or BK receptors caused the inhibition of Ca^{2+} channels via two pathways: the first pathway inhibited the channels rapidly but transiently, and the second followed on and caused sustained inhibition. The pathways appeared to depend on Ca^{2+} in different ways: the first pathway involved a rise in $[Ca^{2+}]_i$ but the second required only the presence of a constant level of $[Ca^{2+}]_i$ – the resting level was sufficient but higher levels of $[Ca^{2+}]_i$ (e.g. from activation of the first pathway) potentiated the effect. The results are also consistent with the view that the first pathway involved the generation of IP_3 and the subsequent release of Ca^{2+} from intracellular stores. The I_{Ca} inhibition appeared to occur absolutely through a G-protein but there was no indication as to whether the G-protein was the same for both pathways.

Evidence for two effects on I_{Ca}

There are several observations that support the view that transient inhibition of I_{Ca} was caused by IP_3-induced Ca^{2+}-release: histamine and BK generated IP_3 in the guinea-pig ileum (Jafferji & Michell, 1975; Ransom *et al.* 1992); heparin blocked transient inhibition of I_{Ca} (Fig. 5); flash photolysis of caged IP_3 caused a transient inhibition of I_{Ca} in smooth muscle cells from the rabbit jejunum (Komori & Bolton, 1991*a*); Ca^{2+}-dependent K^+ currents (Fig. 7) and indo-1 Ca^{2+} measurement (Pacaud & Bolton, 1991) suggested that histamine did cause a transient increase in $[Ca^{2+}]_i$; strong chelation of intracellular Ca^{2+} abolished the effects of histamine and BK (Fig. 3).

Several results suggested that sustained inhibition could occur irrespective of IP_3-induced Ca^{2+} release: $[Ca^{2+}]_i$ was not maintained substantially above the baseline during sustained inhibition (Fig. 7; Pacaud & Bolton, 1991); a very high concentration of intracellular heparin or pretreatment with caffeine did not prevent sustained inhibition; strong buffering of $[Ca^{2+}]_i$ near the resting level with BAPTA–Ca^{2+} mixtures in the pipette permitted sustained but not transient inhibition. In addition, the experiment in Fig. 9*A* suggests that a transient increase in $[Ca^{2+}]_i$ could cause a transient inhibition of I_{Ca} without this being followed by sustained suppression, again suggesting that the two effects could be independent.

Two similar mechanisms for Ca^{2+} channel inhibition may also exist in pituitary tumour (GH_3) cells (Kramer, Kaczmarek & Levitan, 1991). Thyrotropin-releasing hormone seemed to inhibit I_{Ca} in these cells because IP_3 had induced Ca^{2+} release but it was also found that the rise in $[Ca^{2+}]_i$ was transient and the inhibition of I_{Ca} sustained.

On the mechanism for sustained inhibition of I_{Ca}

There are similarities between the mechanism of action of acetylcholine on cationic channels and the actions of histamine and BK on Ca^{2+} channels in guinea-pig ileum smooth muscle cells. Cationic channels seemed to become very sensitive to Ca^{2+} when muscarinic receptors were stimulated – increased $[Ca^{2+}]_i$ increased channel opening (Inoue & Isenberg, 1990*b*; Pacaud & Bolton, 1991). An explanation for this effect could be that activated α-subunits of a G-protein induced or potentiated Ca^{2+} sensitivity in the cationic channels without the need for an additional second messenger. It is possible that an analogous mechanism exists for sustained inhibition of I_{Ca}. Three observations favour such an analogy: sustained inhibition of I_{Ca} was larger and seemed to have a more rapid onset if there was a prior $[Ca^{2+}]_i$ rise (i.e. IP_3-induced Ca^{2+} release); suppression was absent if $[Ca^{2+}]_i$ was chelated to low levels; the transient effect of caffeine on I_{Ca} (probably caused by Ca^{2+} release) appeared greater in the presence of histamine (cf. Figs 4 and 5). However, the intention is not to imply that cationic channels and Ca^{2+} channels were modulated by the same second messenger pathway. Indeed, this seems unlikely because I_{Ca} was inhibited when cationic channels were not activated: histamine caused an inward current at the holding potential (−61 mV) in only sixteen of twenty-nine cells studied and yet I_{Ca} was suppressed in all twenty-nine (Cs^+–low-EGTA pipette solution); BK induced an inward current in only five of fourteen cells and I_{Ca} was suppressed in all fourteen

(results not shown). In addition, there were other distinctions between the two effects which suggested the Ca^{2+} sensitivities arose via different mechanisms. The I_{Ca} was inhibited if $[Ca^{2+}]_i$ was raised by caffeine – this is not true of the cationic channels – and a transient rise in $[Ca^{2+}]_i$ appeared to have a more prolonged effect on Ca^{2+} channels than cationic channels (Fig. 9*A*).

Sustained suppression of I_{Ca} in the ileum seemed to occur by a mechanism that differed from that in smooth muscle cells from the guinea-pig vas deferens where noradrenaline suppressed I_{Ca}, or I_{Ba}, despite the presence of 20 mM EGTA in the recording pipette (Imaizumi *et al.* 1991). However, sustained ATP-induced inhibition of I_{Ca} in the rabbit portal vein did show some Ca^{2+} dependence, which was heparin resistant: suppression was almost absent when there was no extracellular Ca^{2+} and was reduced when the EGTA concentration in the recording pipette was increased from 4 to 20 mM (Xiong, Kitamura & Kuriyama, 1991). Sustained inhibition of I_{Ca} also occurred in response to noradrenaline in rabbit ear artery smooth muscle cells (Droogmans, Declerck & Casteels, 1987; Declerck, Himpens, Droogmans & Casteels, 1990) and rat portal vein cells (Pacaud, Loirand, Mironneau & Mironneau, 1987), and in response to bPTH(1-34), a synthetic fragment of parathyroid hormone, in rat tail artery cells (Wang, Karpinski & Pang, 1991). The Ca^{2+} dependence of these effects was not investigated thoroughly but they all occurred with 10 mM EGTA in the recording pipette.

In the guinea-pig ileum, histamine, BK, acetylcholine and substance P all induce the production of IP_3 (Jafferji & Michell, 1975; Best, Brooks & Bolton, 1985; Ransom *et al.* 1992). They also all caused a sustained suppression of I_{Ca}. This suggests the possibility that IP_3 might not only be involved in the release of stored Ca^{2+} but also in the genesis of the sustained suppression of I_{Ca}. However, this is made unlikely by the observation that sustained suppression was not blocked by 5 mg ml^{-1} heparin, which at 2 mg ml^{-1} blocked all of the effects of IP_3 on I_{Ca} in smooth muscle cells from the rabbit jejunum (Komori & Bolton, 1991*a*). If the messenger was not IP_3 the link with phospholipase C could have occurred via another derivative of phosphatidylinositol 4,5-bisphosphate. One possibility, diacylglycerol, has been suggested to inhibit I_{Ca} in other cell types via an action on protein kinase C. However, although phorbol dibutyrate has been shown to inhibit histamine- and BK-induced contractions in the guinea-pig ileum it did not affect high KCl-induced contractions, suggesting that protein kinase C stimulation did not inhibit Ca^{2+} channels (Baraban, Gould, Peroutka & Snyder, 1985). Therefore, a role for phospholipase C in the sustained suppression of I_{Ca} is not ruled out but the products IP_3 and diacylglycerol seem unlikely intermediates.

Ca^{2+} channel inhibition in the whole tissue

Mitsui & Karaki (1990) showed that carbachol-induced tonic contraction in the guinea-pig ileum became less as the concentration of carbachol was increased above 100 nM, and proposed that 'carbachol may produce endogenous inhibitory substances of the Ca^{2+} channels'. They also suggested that relaxation induced by carbachol in high KCl occurred via a similar mechanism. Histamine and substance P also caused relaxation in high KCl (Bolton *et al.* 1981; Holzer & Lippe, 1984). Therefore, histamine, acetylcholine and substance P may oppose their own spasmogenic actions by inhibiting Ca^{2+} channels. Hall & Bonta (1973) found that BK

relaxed the guinea-pig ileum if it had been contracted by a submaximally effective concentration of acetylcholine, but not if higher concentrations were used. However, they also noted that BK did not cause relaxation if the muscle was contracted with high KCl. This observation is inconsistent with the view that BK inhibited Ca^{2+} channels in the whole tissue and requires further investigation.

Ca^{2+} channel inhibition in the guinea-pig ileum might serve many purposes. Histamine desensitized the ileum to subsequent challenges with acetylcholine (Horio *et al.* 1990; Himpens *et al.* 1991); a non-specific (heterologous) effect which Himpens *et al.* (1991) have suggested might arise because of Ca^{2+} channel block. Ca^{2+} channel inhibition may act to protect cells if there is Ca^{2+} overload. It may allow substances that are normally spasmogenic to cause relaxation if the ileum is already depolarized. Inhibition of Ca^{2+} channels might lead to a suppression of gene expression, as has been shown in cortical neurones (Murphy, Worley & Baraban, 1991). It may also have an excitatory effect by reducing Ca^{2+}-dependent repolarization and after-hyperpolarization of the spike.

This study has shown that spasmogens can inhibit Ca^{2+} channels in single smooth muscle cells from the guinea-pig ileum not simply because they release intracellular Ca^{2+} but because they activate an additional process that can cause pronounced and long-lasting inhibition. In the whole tissue this long-lasting effect may add complexity to the actions of spasmogens and provide a mechanism for their regulation and interaction.

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