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Homologous and heterologous desensitization of histamine H_1- and ATP-receptors in the smooth muscle cell line, DDT_1MF-2: the role of protein kinase C

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1 The possible role of protein kinase C (PKC) in homologous and heterologous desensitization of histamine H_1- and ATP-receptors has been studied in monolayers of cultured vas deferens smooth muscle cells (DDT_1MF-2). Cells were loaded with the calcium-sensitive fluorescent dye fura-2 and increases in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) monitored in response to histamine H_1- or ATP-receptor activation.

2 Histamine and ATP stimulated the release of Ca^{2+} from intracellular Ca^{2+} stores and Ca^{2+} influx across the plasma membrane. Activation of PKC with the phorbol ester β-phorbol-12,13 dibutyrate (PDBu; 1 μM) attenuated histamine (100 μM) and ATP (10 μM)-induced release of intracellular Ca^{2+} and Ca^{2+} influx.

3 The selective PKC inhibitor, Ro 31-8220 (10 μM), reversed the PDBu-induced attenuation of histamine (100 μM)-stimulated Ca^{2+} responses.

4 Histamine H_1- and ATP-receptors are readily susceptible to homologous desensitization since short-term exposure to histamine or ATP (450 s) attenuated the Ca^{2+} responses elicited by a second application of the same agonist. Furthermore, H_1-receptor activation-induced heterologous desensitization of ATP stimulated Ca^{2+} responses and *vice versa*.

5 Homologous and heterologous desensitization of histamine and ATP Ca^{2+} responses still occurred in the presence of the PKC inhibitor, Ro 31-8220 (10 μM).

6 These data suggest that PKC activation can attenuate histamine H_1- and ATP-receptor mediated Ca^{2+} responses. However, based on our experimental data, PKC-independent mechanisms appear to be involved in the homologous and heterologous desensitization of histamine H_1- and ATP-receptor mediated Ca^{2+} responses in DDT_1MF-2 cells.

Keywords: Histamine H_1-receptors; intracellular calcium; ATP; nucleotide receptors; protein kinase C; phorbol esters; Ro 31-8220; desensitization

Introduction

Many cells express receptors that are coupled to phospholipase C, via a regulatory G-protein, which upon activation hydrolyses the plasma-membrane phospholipid, phosphatidylinositol 4,5-biphosphate generating the two second messengers D-*myo*-inositol 1,4,5-trisphosphate ($InsP_3$) and 1,2-diacylglycerol (Berridge, 1993). $InsP_3$ triggers the release of Ca^{2+} from intracellular stores producing a rise in $[Ca^{2+}]_i$, whereas 1,2,-diacylglycerol activates cytosolic protein kinase C (PKC). Tumour-promoting phorbol esters also activate PKC and numerous studies have used phorbol esters to investigate the role of PKC in the regulation of agonist-stimulated inositol phospholipid hydrolysis. For example, PKC activation (using phorbol esters) has been shown to attenuate bombesin (Brown *et al.*, 1987), α_1-adrenoceptor (Woods *et al.*, 1987) and histamine H_1-receptor (Jones *et al.*, 1990) stimulated inositol phospholipid hydrolysis. Based on these findings it has been proposed that agonist-stimulated phospholipase C activity may be desensitized through a negative feedback loop involving the activation of PKC by diacylglycerol. Interestingly, in bovine adrenal chromaffin cells, the selective PKC inhibitor, Ro 31-8220 (Davis *et al.*, 1989) has been shown to enhance the accumulation of $InsP_3$ elicited by histamine and bradykinin (Boarder & Challis, 1992).

The phenomenon of desensitization is characterized by the loss of an agonist-induced response despite the continued presence of the agonist. The mechanism(s) underlying the desensitization of the β-adrenoceptor have been extensively studied (Hausdorff *et al.*, 1990; Palczewski & Benovic, 1991). The β-adrenoceptor is rapidly desensitized by the concerted action of adenosine 3':5'-cyclic monophosphate (cyclic AMP) -dependent protein kinase A (PKA) and cyclic AMP-independent β-adrenoceptors kinase (βARK). Phosphorylation of the β-adrenoceptor causes the uncoupling of the G-protein-receptor complex. In contrast, the mechanism(s) involved in the desensitization of the histamine H_1-receptor are poorly understood. Data obtained from studies monitoring histamine-induced contractions of guinea-pig intestinal smooth muscle suggest that PKC is not involved in homologous desensitization of the histamine H_1-receptor (Leurs *et al.*, 1991). Similarly in HeLa cells, Smit *et al.* (1992) proposed that homologous desensitization of histamine H_1-receptor-mediated intracellular Ca^{2+} release can occur independently of PKC activation and may involve a specific H_1-receptor kinase, whereas heterologous desensitization (studied using ATP) of the H_1-receptor appeared to involve PKC activation.

Homologous desensitization of histamine H_1- and α_1-adrenoceptor-stimulated inositol phospholipid metabolism has been reported in DDT_1MF-2 cells (Cowlen & Toews, 1988; Mitsuhashi & Payan, 1988; Cowlen *et al.*, 1990). Indeed, Leeb-Lundberg *et al.* (1985; 1987) showed that phorbol esters and continued receptor-occupancy promote α_1-adrenoceptor phosphorylation. Our previous studies have shown that in DDT_1MF-2 cells, histamine H_1- and ATP-receptor activation causes the release of Ca^{2+} from intracellular stores ($InsP_3$-induced) and Ca^{2+} entry (influx) across the plasma membrane (Dickenson & Hill, 1991; 1992; Dickenson *et al.*, 1993). Recently, Felder *et al.* (1992) reported that muscarinic M_3-receptor-operated Ca^{2+} influx in transfected A9 fibroblasts is insensitive to phorbol ester pretreatment, whereas Ca^{2+} re-

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lease was inhibited by PKC activation. Similarly, in PC12-64 cells, PKC activation affected carbachol-stimulated intracellular Ca^{2+} release more than Ca^{2+} influx (Clementi *et al.*, 1992).

Therefore, the aims of the present study were: (i) to determine if phorbol esters could selectively inhibit histamine H_1- or ATP-receptor stimulated intracellular Ca^{2+} release or Ca^{2+} influx and (ii) to investigate the role of PKC, using the selective PKC inhibitor, Ro 31-8220 (Davis *et al.*, 1989), in homologous and heterologous desensitization of histamine H_1- and ATP-receptor-mediated Ca^{2+} responses in the smooth muscle cell line, DDT_1MF-2.

Methods

Cell culture

The hamster vas deferens smooth muscle cell line (DDT_1MF-2) was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, Wiltshire). DDT_1 MF-2 cells were cultured at 37°C in a humidified air/CO_2 (90:10) atmosphere in 75 cm^2/flasks (Costar). The growth medium was Dulbecco's modified Eagle's medium supplemented with 2 mM-L-glutamine and 10% (v/v) foetal calf serum (FCS). Cells were passaged twice a week (1/5 split ratio) by vigorous shaking of the flask and placed into 75 cm^2 flasks and fed with fresh growth medium every 48 h. Cells for $[Ca^{2+}]_i$ determinations were grown on 24 mm × 10 mm glass coverslips in 90 mm petri dishes. All experiments were performed on confluent monolayers (passages 4–21, numbers assigned after receiving the cell line).

Measurement of intracellular free calcium

Intracellular free calcium was measured by loading confluent cell monolayers with the calcium-sensitive fluorescent dye fura-2. Individual coverslips were placed in 35 mm petri dishes with 1 ml of physiological buffer (composition, mM: NaCl 145, glucose 10, KCl 5, $MgSO_4$ 1, HEPES 10, $CaCl_2$ 2, pH 7.45) containing 10% FCS (v/v), 3 μM fura-2/AM and incubated for 30 min at 37°C. After this 'loading' period, the fura-2 containing buffer was replaced with fresh buffer that was free of fura-2 and FCS but contained 0.1% bovine serum albumin, and left at 37°C for a further 15 min. Loaded coverslips were then mounted in a specially designed holder which enabled the coverslip to be positioned across the diagonal of a polymethacrylate cuvette. Each cuvette contained 2.9 ml of physiological buffer (drugs were added to the cuvettes in 100 μl aliquots) and fluorescent measurements were made at 37°C in a Perkin Elmer LS 50 spectrometer. The excitation wavelengths were 340 and 380 nm, with emission at 500 nm. The slit-widths were set at 10 nm for both the excitation and emission wavelengths and the time taken to switch between 340 and 380 nm was 0.8 s. Intracellular Ca^{2+} was calculated every 1.9 s from the ratio (R) of 340 nm/380 nm fluorescent values using the equation of Grynkiewicz *et al.* (1985).

$$[Ca^{2+}]_i = \frac{(R-R_{min})}{(R_{max.}-R)} \times (S_{380,min.}/S_{380,max.}) \times K_d$$

where K_d is the affinity of fura-2 for Ca^{2+} (224 nM at 37°C) and $S_{380,min.}/S_{380,max.}$ is the ratio (β value) of the fluorescent values obtained at 380 nm in the absence and presence of saturating $[Ca^{2+}]_i$. The maximum and minimum R values ($R_{max.}$ and $R_{min.}$) were determined on separate coverslips under saturating $[Ca^{2+}]_i$ (achieved by increasing the extracellular $[Ca^{2+}]$ to 20 mM followed by 10 μM ionomycin, pH 7.4) and calcium-free (achieved using 8.3 mM EGTA immediately followed by 25 μl of 1.0 M NaOH to compensate for the decrease in pH, in the presence of 10 μM ionomycin) conditions respectively. Corrections for autofluorescence were made by measuring the fluorescence produced by coverslips

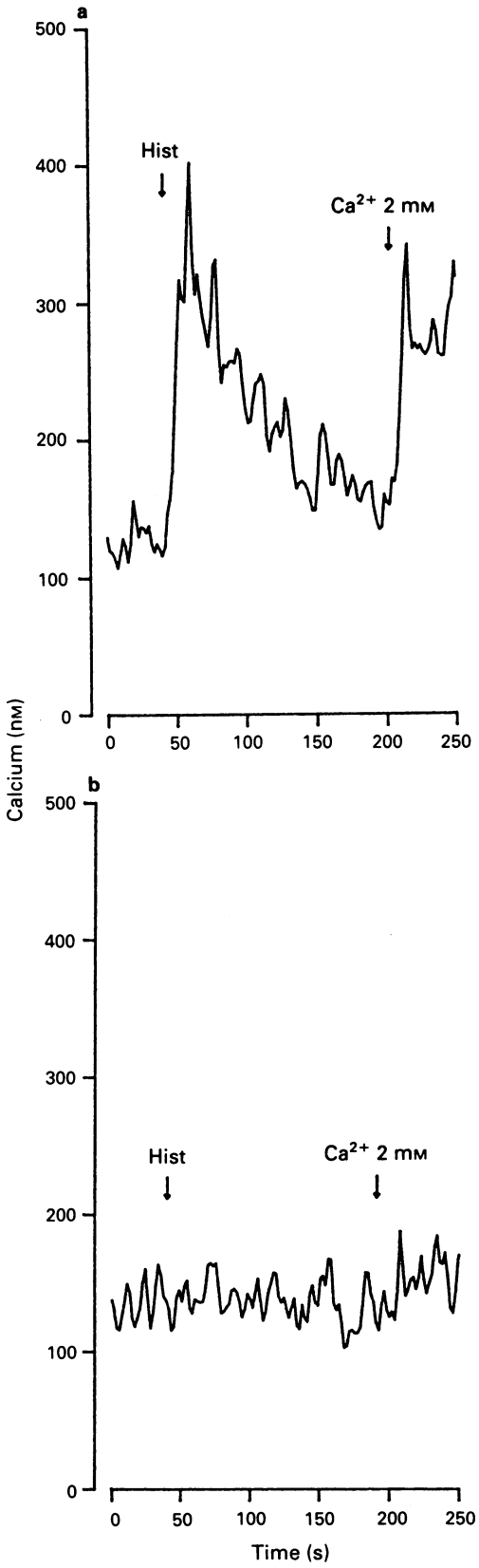

Figure 1 Effect of β-phorbol-12,13 dibutyrate on histamine H_1-receptor mediated increases in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. (a) Cells were stimulated with histamine (Hist) in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA after which exogenous Ca^{2+} (2 mM) was re-applied. (b) The same protocol as in (a), but cells were preincubated for 15 min with 1 μM β-phorbol-12,13-dibutyrate (PDBu). Experiment (a) contained 0.01% dimethyl sulphoxide to control for PDBu vehicle effects. Histamine (100 μM) and $CaCl_2$ (2 mM) were added where indicated. Similar results were obtained in eight other experiments.

that had not been loaded with fura-2. Where Ca^{2+}-free conditions were required experiments were performed in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA.

Data analysis

Rises in intracellular free $[Ca^{2+}]$ were evaluated by importing the fluorescence data into the spreadsheet AsEasyAs (TRIUS Inc). Basal $[Ca^{2+}]_i$ levels were determined by calculating the mean of the ten data points (measured every 1.9 s) prior to drug addition, whereas the maximum Ca^{2+} signal was deemed to be the largest Ca^{2+} response obtained immediately after drug addition. Due to the fluctuations in basal $[Ca^{2+}]_i$, the mean of the ten data points measured immediately after drug addition were also calculated to determine whether the measured response was significantly different from basal levels (Mann Whitney U test). Data are shown as means ± s.e.mean. Statistical analysis was performed with Student's unpaired t test. A P value <0.05 was considered as statistically significant; n in the text refers to the number of separate experiments.

Chemicals

Fura-2/AM and ionomycin were from Calbiochem. Histamine, bradykinin, adenosine-5'-triphosphate, β-phorbol-12, 13 dibutyrate, and 4α-phorbol were obtained from Sigma Chemical Company Ltd (U.K.). Ro 31-8220 (3-{1-[3-(2-isothioureido)propyl]indol-3-yl}-4-(1-methylindol-3-yl)-3-pyrrolin-2,-5-dione) was a kind gift from Dr T. Hallam, Roche Products Ltd. (Welwyn Garden City, Herts). Dulbecco's modified Eagle's medium and foetal calf serum (FCS) were from Northumbria Biologicals (U.K.). All other chemicals were of analytical grade.

Results

Effect of β-phorbol-12,13 dibutyrate on histamine H_1- and ATP-receptor stimulated increases in $[Ca^{2+}]_i$

Experiments monitoring agonist-stimulated increases in $[Ca^{2+}]_i$ were carried out using the Ca^{2+}-free/Ca^{2+}-reintroduction protocol. This involved initially stimulating the cells in Ca^{2+}-free buffer containing 0.1 mM EGTA (to measure intracellular Ca^{2+} release) after which extracellular Ca^{2+} (2 mM) was re-applied (to measure Ca^{2+} influx). Figure 1a shows histamine (100 μM)-stimulated increases in $[Ca^{2+}]_i$ in DDT_1MF-2 cells. Ca^{2+} release increased $[Ca^{2+}]_i$ from 111 ± 6 nM to 408 ± 15 nM ($n = 8$), whereas Ca^{2+} influx increased $[Ca^{2+}]_i$ from 139 ± 9 nM to 390 ± 18 nM ($n = 8$). PDBu pretreatment (1 μM; 15 min) completely abolished H_1-receptor stimulated intracellular Ca^{2+} release and influx ($n = 8$; see Figure 1b). In contrast, the Ca^{2+} responses elicited by maximal concentrations of ATP (100 μM) were insensitive to PDBu pretreatment. The maximum peak Ca^{2+} responses to ATP (100 μM) obtained in Ca^{2+}-free buffer and upon the addition of extracellular Ca^{2+} (2 mM) in PDBu-treated cells were 98 ± 4% ($n = 11$) and 102 ± 6% ($n = 11$) respectively, of those obtained in control experiments (see Table 1). However, if the concentration of ATP was reduced to 10 μM, PDBu pretreatment significantly reduced ATP-induced Ca^{2+} responses (Figure 2). In these experiments the maximum peak Ca^{2+} responses to ATP (10 μM) obtained in Ca^{2+}-free buffer and upon the addition of exogenous Ca^{2+} in PDBu-treated cells were 45 ± 8% ($n = 11$) and 35 ± 5% ($n = 11$) respectively, of those obtained in control experiments (see Table 1). Pretreatment with inactive phorbol 4α-phorbol (1 μM, 15 min) did not inhibit histamine (100 μM)-stimulated intracellular Ca^{2+} release ($[Ca^{2+}]_i$ increased from 108 ± 7 nM to 420 ± 19 nM in control cells and from 103 ± 9 nM to 408 ± 12 nM in 4α-phorbol treated cells; $n = 5$) or Ca^{2+} influx ($[Ca^{2+}]_i$ increased from 125 ± 12 nM to 320 ± 11 nM in control cells and from 118 ± 6 nM to 340 ± 17 nM in 4α-phorbol treated cells; $n = 5$).

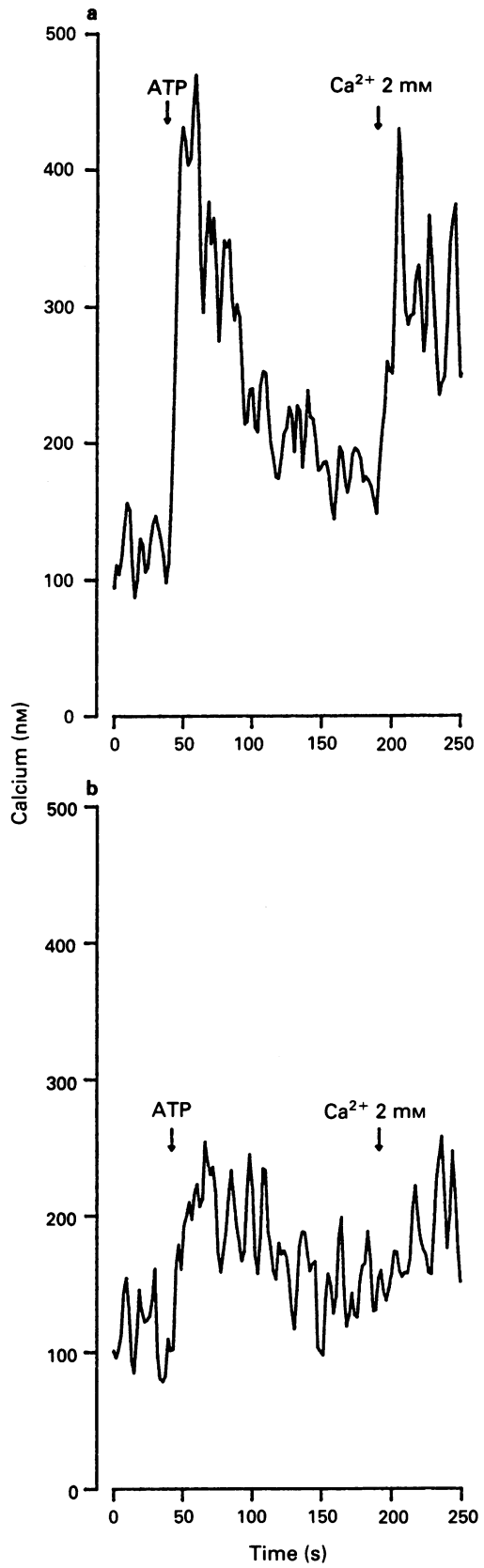

Figure 2 Effect of β-phorbol-12,13 dibutyrate on ATP-receptor mediated increases in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. (a) Cells were stimulated with ATP in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA after which exogenous Ca^{2+} (2 mM) was re-applied. (b) The same protocol as in (a), but cells were preincubated for 15 min with 1 μM β-phorbol-12,13-dibutyrate (PDBu). Experiment (a) contained 0.01% dimethyl sulphoxide to control for PDBu vehicle effects. ATP (10 μM) and $CaCl_2$ (2 mM) were added where indicated. Similar results were obtained in ten other experiments.

Table 1 The effect of β-phorbol-12,13-dibutyrate on ATP-receptor stimulated increases in intracellular calcium

	100 μM ATP			*10 μM ATP*		
	Basal $[Ca^{2+}]_i$ (nM)	*Stimulated*	(*n*)	*Basal* $[Ca^{2+}]_i$ (nM)	*Stimulated*	(*n*)
Ca^{2+} release						
Control	115 ± 6	596 ± 20	11	123 ± 8	450 ± 18	11
PDBu 1 μM	108 ± 9	570 ± 15	11	116 ± 7	263 ± 13*	11
Ca^{2+} influx						
Control	121 ± 8	409 ± 12	11	150 ± 12	435 ± 20	11
PDBu 1 μM	118 ± 6	421 ± 9	11	161 ± 9	270 ± 13*	11

Values represent the mean ± s.e.mean of (*n*) experiments. Statistical analysis was performed (Student's unpaired *t* test) comparing the stimulated Ca^{2+} responses (after subtracting basal $[Ca^{2+}]_i$) obtained in control and treated cells. *Statistically significant, $P<0.05$, from control responses.

Effect of protein kinase C inhibitor Ro 31-8220 on histamine H_1-receptor mediated increases in $[Ca^{2+}]_i$

Preincubation with 500 nM PDBu abolished the histamine (100 μM)-stimulated Ca^{2+} response measured in the presence of extracellular Ca^{2+} (compare Figure 3a with Figure 3b). Pretreatment with Ro 31-8220 (10 μM; 45 min) had no effect on the histamine response (95 ± 7% of control; $n = 6$; Figure 3c). Finally, Ro 31-8220 completely reversed the PDBu-induced attenuation of histamine (100 μM)-stimulated increases in $[Ca^{2+}]_i$ (96 ± 9% of controls; $n = 6$; Figure 3d).

Homologous desensitization of histamine H_1- and ATP-receptor stimulated increases in $[Ca^{2+}]_i$

We performed a series of experiments to study the homologous desensitization of histamine H_1- and ATP-receptor-mediated Ca^{2+} responses in DDT_1MF-2 cells. The experimental protocol involved initially stimulating the cells with maximally-effective concentrations of either agonist in nominally Ca^{2+}-free buffer (for approximately 150 s) after which extracellular Ca^{2+} (2 mM) was added for 300 s in the continued presence of agonist (to allow refilling of the intracellular Ca^{2+} stores). After 500 s the glass coverslip was transferred to a fresh cuvette that contained Ca^{2+}-free buffer (plus 0.1 mM EGTA) before monitoring the Ca^{2+} responses elicited by a second application of the appropriate agonist. Figure 4a shows that short term exposure (450 s) to histamine (100 μM) induces homologous desensitization of histamine H_1-receptor-mediated intracellular Ca^{2+} release and Ca^{2+} influx. An appropriate control for Figure 4a is shown in Figure 4b. In these experiments the cells were briefly exposed to histamine in Ca^{2+}-free buffer (for approximately 150 s) after which the glass coverslip was transferred to a fresh cuvette that contained Ca^{2+}-containing buffer (in the absence of agonist). As we have reported previously in these cells (Dickenson & Hill, 1992) the removal of histamine from its receptor (with mepyramine) prevents Ca^{2+} influx into the cytoplasm which is dependent upon H_1-receptor occupancy (receptor-mediated Ca^{2+} influx) but allows the refilling of the intracellular Ca^{2+} stores (independent of receptor occupancy). In these experiments the second addition of histamine resulted in intracellular Ca^{2+} release (86 ± 6% of the first application; $n = 8$) and Ca^{2+} influx (114 ± 4% of controls; $n = 8$). These data

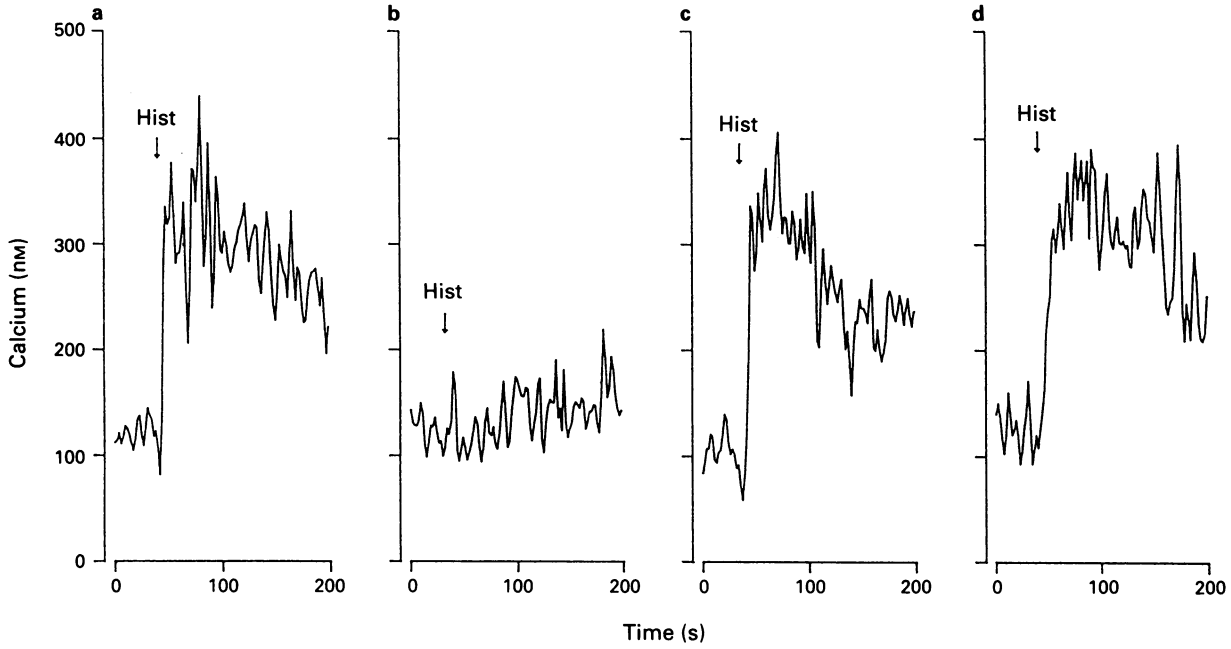

Figure 3 Effect of the selective protein kinase C inhibitor, Ro 31-8220, on β-phorbol-12,13-dibutyrate-induced attenuation of histamine H_1-receptor mediated Ca^{2+} responses. (a) Histamine stimulated increases in $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} (2 mM). (b) As in (a) but cells were preincubated for 15 min with 500 nM β-phorbol-12,13-dibutyrate (PDBu). (c) Histamine stimulated Ca^{2+} response in the presence of 10 μM Ro 31-8220 (45 min preincubation). (d) Cells incubated for 30 min with 10 μM Ro 31-8220 followed by 15 min with 10 μM Ro 31-8220 in the presence of 500 nM PDBu. Experiments (a) to (d) contained 0.01% dimethyl sulphoxide to control for PDBu and Ro 31-8220 vehicle effects. Histamine (Hist, 100 μM) was added where indicated. Similar results were obtained in five other experiments.

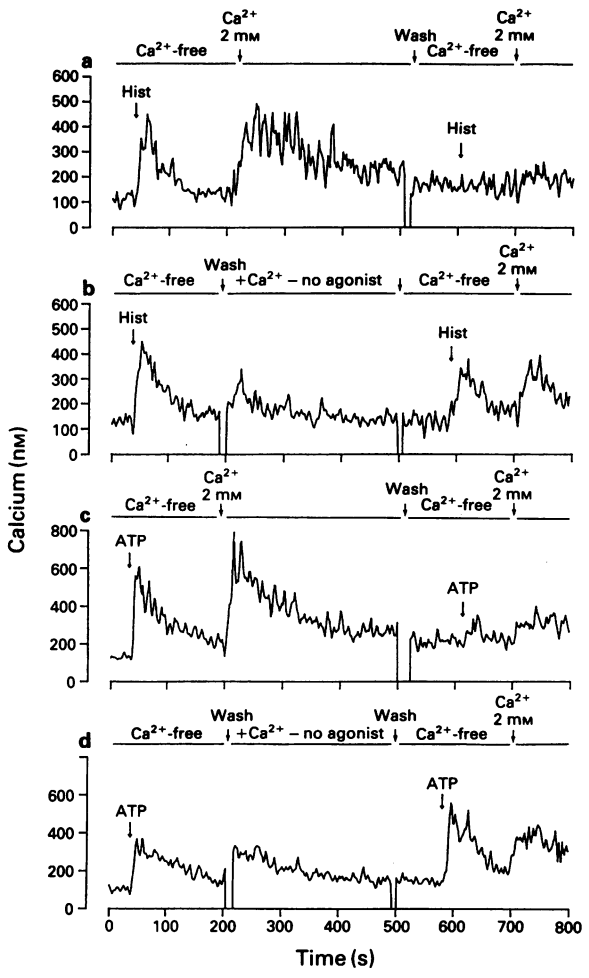

Figure 4 Homologous desensitization of histamine H_1- and ATP-receptor mediated increases in $[Ca^{2+}]_i$ in DDT_1MF-2 cells. (a) Homologous desensitization of histamine H_1-receptor mediated Ca^{2+} responses. (b) Control experiment for (a) to eliminate the possibility that the absence of a second histamine response is not simply a consequence of the intracellular Ca^{2+} stores being empty. (c) Homologous desensitization of ATP-receptor-mediated Ca^{2+} responses. (d) Control experiment for (c) using the same experimental design as in (b). Histamine (Hist, 100 μM), ATP (100 μM) or $CaCl_2$ (2 mM) were added where indicated. During Ca^{2+}-free periods buffer also contained 0.1 mM EGTA. Similar results were obtained in five other experiments.

suggest that the absence of a second histamine response in Figure 4a is due to homologous desensitization and not a consequence of intracellular Ca^{2+} store depletion or experimental protocol. Furthermore, our previous studies with bradykinin (which does not exhibit heterologous desensitization following H_1-receptor activation) have shown that intracellular Ca^{2+} stores are able to refill in the continued presence of histamine and 2 mM Ca^{2+} (cf. Figure 6c in Dickenson & Hill, 1992).

Similar experiments were performed to determine whether ATP-mediated Ca^{2+} responses are susceptible to homologous desensitization. Figure 4c shows the Ca^{2+} responses obtained by a second application of ATP (100 μM; same protocol as described for Figure 4a) after an initial 450 s exposure to ATP. Both intracellular Ca^{2+} release (11 ± 4% of the first response; $n = 7$) and Ca^{2+} influx (20 ± 7% of the first response; $n = 7$) were significantly attenuated. The relevant control experiment is shown in Figure 4d. In the control experiment the second application of ATP resulted in optimal Ca^{2+} responses for intracellular Ca^{2+} release (96 ± 8% of the first application; $n = 4$) and Ca^{2+} influx (90 ± 7% of controls; $n = 4$).

Heterologous desensitization of histamine H_1- and ATP-receptor-stimulated increases in $[Ca^{2+}]_i$

Having established that both histamine and ATP-receptor mediated Ca^{2+} responses undergo homologous desensitization we investigated the effects of histamine pretreatment on ATP-responses and *vice versa*. Pretreatment with histamine (450 s) markedly reduced the Ca^{2+} responses elicited by ATP (100 μM; Figure 5a). The Ca^{2+} responses attributed to ATP-mediated intracellular Ca^{2+} release and Ca^{2+} influx were 40 ± 6% ($n = 12$) and 52 ± 7% ($n = 12$) respectively, of those obtained in control experiments (see Figure 5b). Similarly, pretreatment with ATP (450 s) significantly attenuated histamine-induced Ca^{2+} responses (Figure 5c). In these experiments histamine-stimulated intracellular Ca^{2+} release and Ca^{2+} influx responses were 32 ± 7% ($n = 12$) and 52 ± 3% ($n = 12$) respectively, of those obtained in control experiments (see Figure 5d). We have previously shown that short term exposure to histamine did not desensitize the response to bradykinin (Dickenson & Hill, 1992). Short term exposure to bradykinin (1 μM; 450 s) also had no effect on histamine (100 μM) Ca^{2+} responses (intracellular Ca^{2+} release 93 ± 4% of controls; Ca^{2+} influx 89 ± 5% of controls, $n = 10$).

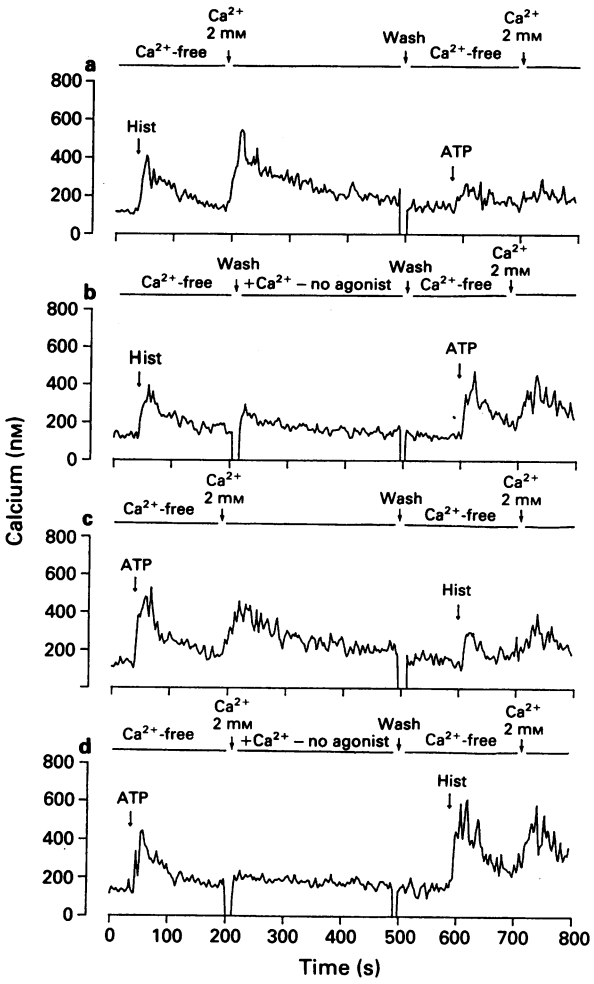

Figure 5 Heterologous desensitization of histamine H_1- and ATP-receptor mediated increases in $[Ca^{2+}]_i$ in DDT_1MF-2 cells. (a) Histamine-induced heterologous desensitization of ATP-receptor responses. (b) Control experiment for (a) to eliminate the possibility that the diminished ATP response is simply a consequence of the intracellular Ca^{2+} stores being empty. (c) ATP induced heterologous desensitization of histamine H_1-receptor Ca^{2+} responses. (d) Control experiment for (c) using the same experimental design as in (b). Histamine (Hist, 100 μM), ATP (100 μM) or $CaCl_2$ (2 mM) were added where indicated. Similar results were obtained in five other experiments.

Effect of Ro 31-8220 on homologous desensitization of histamine H_1- and ATP-receptor-stimulated increases in $[Ca^{2+}]_i$

To assess the possible involvement of PKC in homologous desensitization of histamine H_1- and ATP-receptor-mediated Ca^{2+} responses, PKC activity was blocked by preincubating DDT_1MF-2 cells with the selective PKC inhibitor, Ro 31-8220. Figure 6a shows homologous desensitization of histamine-stimulated Ca^{2+} responses. The complete desensitization of histamine Ca^{2+} responses was still observed in cells pretreated with Ro 31-8220 (10 μM; 45 min; see Figure 6b). Similarly, preincubation with Ro 31-8220 had no effect on the homologous desensitization of ATP-receptor mediated Ca^{2+} responses (compare Figure 6c with 6d). In these experiments the short-term exposure to ATP (450 s) completely abolished the response to a second ATP challenge (this occurred in approximately 50% of experiments). These data suggest that the homologous desensitization of histamine H_1- and ATP-receptor-mediated Ca^{2+} responses can occur independently of PKC activation.

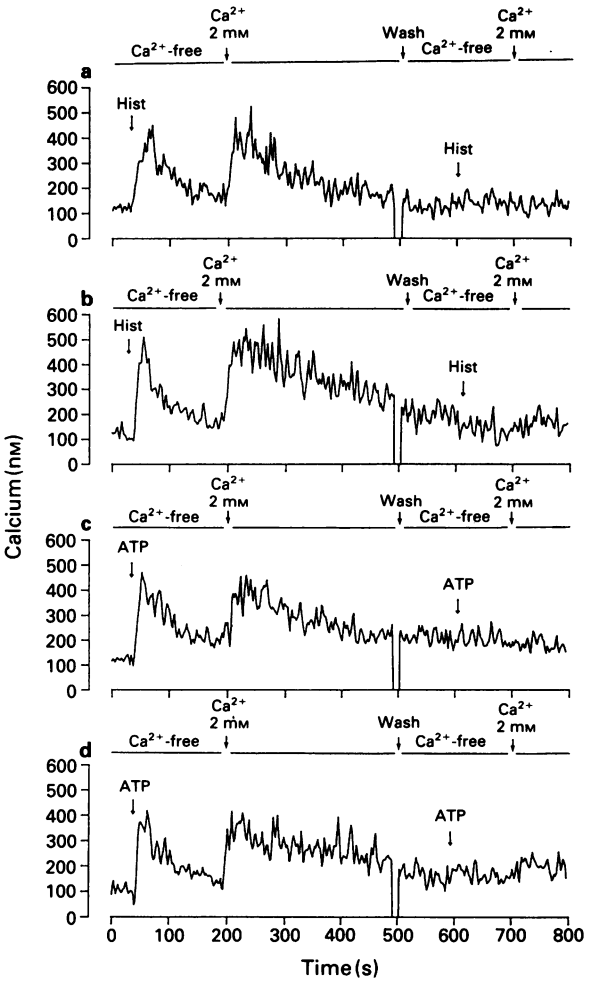

Figure 6 The effect of the protein kinase C inhibitor Ro 31-8220 on homologous desensitization of histamine H_1- and ATP-receptor mediated increases in $[Ca^{2+}]_i$ in DDT_1MF-2 cells. In experiments (b) and (d) the protein kinase C inhibitor Ro 31-8220 (10 μM) was present throughout. (a) Homologous desensitization of histamine H_1-receptor mediated Ca^{2+} responses. (b) The same protocol as in (a), but cells had been preincubated for 45 min with Ro 31-8220 (10 μM). (c) Homologous desensitization of ATP-receptor-mediated Ca^{2+} responses. (d) The same protocol as in (c), but cells had been preincubated for 45 min with Ro 31-8220 (10 μM). Experiments (a) and (c) contained 0.01% dimethyl sulphoxide to eliminate Ro 31-8220 vehicle effects. Histamine (Hist, 100 μM), ATP (100 μM) or $CaCl_2$ (2 mM) were added where indicated. Similar results were obtained in seven other experiments.

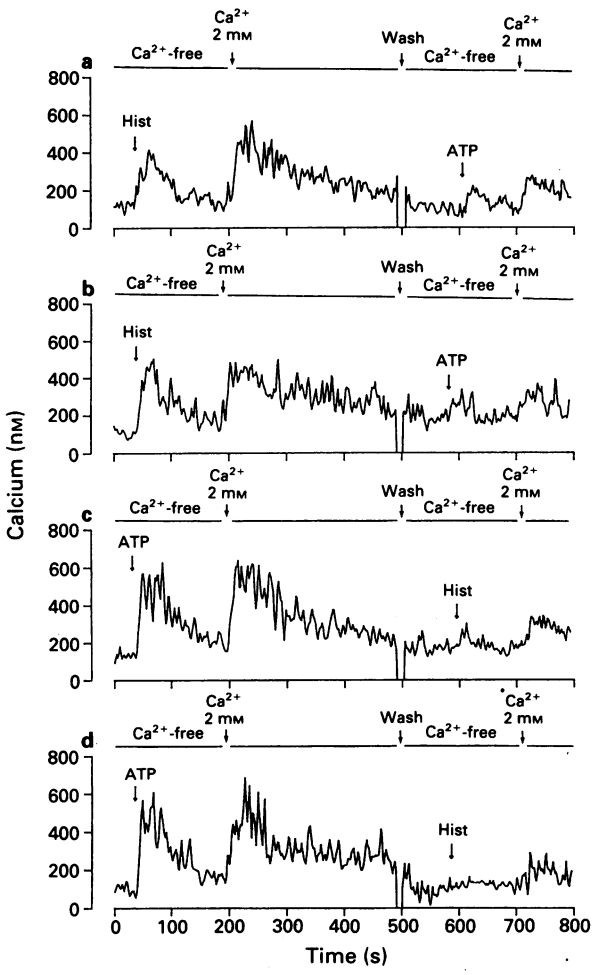

Figure 7 The effect of the protein kinase C inhibitor Ro 31-8220, on heterologous desensitization of histamine H_1- and ATP-receptor mediated increases in $[Ca^{2+}]_i$ in DDT_1MF-2 cells. In experiments (b) and (d) the protein kinase C inhibitor, Ro 31-8220 (10 μM) was present throughout. (a) Histamine induced heterologous desensitization of ATP Ca^{2+} responses. (b) The same protocol as in (a), but cells were preincubated with Ro 31-8220 (10 μM) for 45 min. (c) ATP induced heterologous desensitization of histamine Ca^{2+} responses. (d) The same protocol as in (c), but cells had been preincubated with Ro 31-8220 (10 μM) for 45 min. Experiments (a) and (c) contained 0.01% dimethyl sulphoxide to eliminate Ro 31-8220 vehicle effects. Histamine (Hist, 100 μM), ATP (100 μM) or $CaCl_2$ (2 mM) were added where indicated. Similar results were obtained in seven other experiments.

Effect of Ro 31-8220 on heterologous desensitization of histamine H_1- and ATP-receptor stimulated increases in $[Ca^{2+}]_i$

Recently, Smit *et al.* (1992) hypothesised that in HeLa cells the heterologous desensitization (studied using ATP) of H_1-receptor-mediated increases in $[Ca^{2+}]_i$ (predominantly Ca^{2+} influx) may involve PKC activation. Figure 7b shows that histamine-induced heterologous desensitization of ATP stimulated Ca^{2+} responses still occurred in Ro 31-8220 (10 μM; 45 min)-treated cells (compare Figure 7a with 7b). Similarly, preincubation with Ro 31-8220 had no effect on ATP-induced heterologous desensitization of histamine-mediated Ca^{2+} responses (compare Figure 7c with 7d). It should be noted that ATP-receptor-mediated Ca^{2+} responses are unaffected by Ro-31-8220 pretreatment (intracellular Ca^{2+} release 122 ± 7% of controls; Ca^{2+} influx 103 ± 6% of controls; $n = 8$). The data in Table 2 summarize the effect of Ro 31-8220 on histamine and ATP-receptor heterologous desensitization.

Table 2 The effect of the protein kinase C inhibitor, Ro 31-8220, on heterologous desensitization of histamine and ATP-receptor mediated increases in $[Ca^{2+}]_i$

	Untreated (% of control Ca^{2+} responses)	(*n*)	*Ro 31-8220* (% of control Ca^{2+} responses)	(*n*)
ATP (100 μM)				
Ca^{2+} release	41 ± 8	8	16 ± 9	8
Ca^{2+} influx	45 ± 6	8	46 ± 8	8
Histamine (100 μM)				
Ca^{2+} release	16 ± 9	8	3 ± 2	8
Ca^{2+} influx	48 ± 4	8	41 ± 3	8

Values represent heterologous desensitization of Ca^{2+} responses for histamine H_1- and ATP-receptor stimulation in control and Ro 31-8220 (10 μM)-treated cells (obtained after the initial stimulation with ATP or histamine respectively) and are expressed as a percentage of the Ca^{2+} responses elicited by histamine and ATP-receptor stimulation in control experiments, i.e. the same protocol as that used for histamine and ATP Ca^{2+} responses in Figures 5d and 5b respectively. Values represent the mean ± s.e.mean of (*n*) experiments.

Discussion

In this study we have shown that histamine H_1- and ATP-receptor-mediated increases in $[Ca^{2+}]_i$ are susceptible to homologous and heterologous desensitization (see Figures 4 and 5). However, the mechanisms involved in histamine H_1-receptor desensitization are poorly understood. Therefore, one of the aims of this work was to investigate the possible role PKC may have in agonist-induced desensitization of the histamine H_1-receptor.

The data presented in this study have shown that in DDT_1MF-2 cells, histamine H_1- and ATP-receptor-mediated increases in $[Ca^{2+}]_i$ (both intracellular Ca^{2+} release and Ca^{2+} influx) can be inhibited by PKC activation (with the phorbol ester PDBu). Furthermore, using the selective PKC inhibitor Ro 31-8220, we were able to reverse the inhibitory effects of PDBu on agonist-stimulated increases in $[Ca^{2+}]_i$, confirming the involvement of PKC in these events. The recent cloning of both the bovine (Yamashita *et al.*, 1991) and rat (Fujimoto *et al.*, 1993) histamine H_1-receptor has revealed several potential PKC phosphorylation sites in the amino acid sequence of the H_1-receptor. The availability of a selective PKC inhibitor, Ro 31-8220 (Davis *et al.*, 1989) has enabled us to investigate the role of PKC in the desensitization of histamine H_1- or ATP-receptor Ca^{2+} responses. The data presented show that homologous (see Figure 6) and heterologous (see Figure 7) desensitization of histamine and ATP-receptor-mediated increases in $[Ca^{2+}]_i$ still occurred in the presence of Ro 31-8220. Therefore, based on our experimental data, it would appear that PKC is not involved in either the homologous or heterologous desensitization of these receptors.

These data are similar to those recently reported in HeLa cells (Smit *et al.*, 1992) where, although the H_1-receptor-mediated increases in $[Ca^{2+}]_i$ (intracellular Ca^{2+} release and Ca^{2+} influx) are phorbol ester-sensitive, the homologous desensitization of H_1-receptor-mediated intracellular Ca^{2+} release appears to involve a mechanism which is PKC-independent. Similarly this group (Leurs *et al.*, 1991) have suggested that PKC is not involved in the homologous desensitization of H_1-receptor-induced contractions of guinea-pig intestinal smooth muscle (again phorbol ester-sensitive). In contrast to the data presented in this paper, Smit *et al.* (1992) also proposed that the non-selective inhibition (heterologous desensitization) of histamine and ATP-receptor-mediated Ca^{2+} influx may involve a PKC-dependent mechanism. These observations and the data presented in this paper suggest that, although initial H_1-receptor-mediated functional responses (i.e. contractions, Ca^{2+} release) are phorbol ester-sensitive, PKC-independent mechanisms appear to be involved in the homologous desensitization of the H_1-receptor.

Extensive research has shown that β-adrenoceptor desensitization is mediated by PKA and a receptor-specific β-adrenoceptor kinase (βARK; Hausdorff *et al.*, 1990; Palczewski & Benovic, 1991). Our previous studies (Dickenson *et al.*, 1993) have shown that the activation of cyclic AMP-dependent protein kinase A (PKA; achieved by elevating cytosolic cyclic AMP levels with forskolin) in DDT_1MF-2 cells attenuates histamine H_1-receptor-mediated inositol phospholipid hydrolysis and Ca^{2+} mobilization (the ATP responses are unaffected by PKA). Furthermore, in DDT_1MF-2 cell monolayers prelabelled with $[^3H]$-adenine, both histamine and ATP failed to stimulate the accumulation of $[^3H]$-cyclic AMP (data not shown). These data suggest that PKA is not involved in the desensitization of histamine H_1- or ATP-mediated Ca^{2+} responses.

There are several further possible mechanisms by which the desensitization of histamine and ATP Ca^{2+} responses is occurring. One explanation is that the $InsP_3$ receptor is being desensitized (possibly via autophosphorylation; Ferris *et al.*, 1992), this would account for both homologous and heterologous desensitization (assuming histamine and ATP release Ca^{2+} from the same intracellular Ca^{2+} store). However, the finding that bradykinin does not desensitize histamine Ca^{2+} responses and *vice versa* (Dickenson & Hill, 1992) suggest that $InsP_3$ receptor desensitization is not occurring. These data would also seem to rule out the possibility that a calcium-calmodulin dependent kinase is involved. An alternative explanation is that receptor-activated kinases may be involved in the homologous desensitization of histamine and ATP response, analogous to the βARK. Interestingly, it has been shown that in DDT_1MF-2 cells, β-adrenoceptor activation leads to the translocation of βARK activity from the cytosol to the plasma membrane (Strasser *et al.*, 1986). However to date, there is little evidence for the existence of other G-protein-coupled receptor kinases. If a histamine H_1-receptor specific kinase is responsible for homologous desensitization, the histamine-induced heterologous desensitization of ATP responses (and *vice versa*) may be due to the absence of receptor kinase specificity. The βARK has been shown to phosphorylate other G-protein coupled receptors, for example α_2-adrenoceptor and muscarinic M_2-receptors (Benovic *et al.*, 1987; Kwatra *et al.*, 1989). Smit *et al.* (1992) have also proposed that the homologous desensitization of the H_1-receptor in HeLa cells is associated with activation of a H_1-receptor kinase.

In conclusion, the present study has shown that PKC activation can inhibit histamine H_1- and ATP-receptor mediated Ca^{2+} responses (intracellular Ca^{2+} release and Ca^{2+} influx). However, PKC activation does not appear to be involved in homologous or heterologous desensitization of histamine or ATP-receptor mediated increases in $[Ca^{2+}]_i$.

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