Biochemical Pharmacology, Vol. 46, No. 4, pp. 583–588, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

INHIBITION BY Ro 31-8220 OF ACID SECRETORY ACTIVITY INDUCED BY CARBACHOL INDICATES A STIMULATORY ROLE FOR PROTEIN KINASE C IN THE ACTION OF MUSCARINIC AGONISTS ON ISOLATED RAT PARIETAL CELLS

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(*Received* 16 *March* 1993; *accepted* 24 *May* 1993)

Abstract—The bisindolylmaleimide Ro 31-8220 is a selective inhibitor of protein kinase C. This compound was used to investigate the involvement of protein kinase C in the stimulation of gastric acid secretion by the muscarinic cholinergic receptor on rat isolated parietal cells. The accumulation of the weak base aminopyrine by both crude and enriched preparations of parietal cells was used as an index of secretory activity. Ro 31-8220 antagonized (IC_{50}, 1.0 μM) the effect of the activator of protein kinase C, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), on histamine-stimulated aminopyrine accumulation. Ro 31-8220 (0.1–2.14 μM) inhibited the aminopyrine response to 0.1 mM carbachol (IC_{50}, 0.78 μM; 49% inhibition at 2.14 μM Ro 31-8220) and shifted the dose–response curve for the effect of carbachol concentration of aminopyrine accumulation downwards and to the right. No inhibition of aminopyrine accumulation induced by histamine was found with Ro 31-8220 (0.1–2.14 μM). In a preparation containing >80% parietal cells incubated with 0.1 mM carbachol, 2.14 μM Ro 31-8220 inhibited aminopyrine accumulation by 43%, but had no effect on the increase in intracellular Ca^{2+} which was measured by using the fluorescent probe FURA-2. In conclusion, Ro 31-8220 (0.1–2.14 μM) produced a selective reduction in secretory activity in parietal cells by inhibition of protein kinase C. The predominant role of protein kinase C in parietal cells activated with carbachol is not to cause feedback inhibition of the response but to facilitate stimulation of secretory activity.

Acetylcholine is an important physiological stimulant of gastric acid secretion [1]. Acetylcholine activates a muscarinic receptor on the parietal cell which is of the M_3 type [2, 3]. Stimulation of this receptor by carbachol induces the breakdown of phosphatidylinositol 4,5-bisphosphate to produce inositol 1,4,5-trisphosphate [2, 4, 5] and diacylglycerol [6] in parietal cells. The rise in inositol 1,4,5-trisphosphate is coupled to an elevation of intracellular Ca^{2+} [7–9], while the increase in diacylglycerol is associated with the translocation of protein kinase C [10] from the cytosol to the membrane [4] and its presumed activation [10].

Elevation of intracellular Ca^{2+} appears to be obligatory for stimulation of acid secretion by carbachol [7, 9], and the effects of Ca^{2+} probably involve activation of Ca^{2+}/calmodulin-dependent protein kinase II [11]. The role of protein kinase C in the response to carbachol is much less clear. Some results imply that protein kinase C could be responsible for a feedback inhibitory regulation of the muscarinic receptor. Thus, when protein kinase C is activated in parietal cells by the addition of the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA†), the secretory activity in response to carbachol is inhibited [4, 12, 13]. Protein kinase C may phosphorylate the muscarinic receptor [14] and lead to its uncoupling from phospholipase C with concomitant reduction in the release of inositol phosphates by carbachol [4, 5]. Further support for an inhibitory role for protein kinase C comes from the inhibition of carbachol-stimulated secretory activity in parietal cells on elevation of diacylglycerol by the diacylglycerol lipase inhibitor RHC 80267. Interpretation of these results is, however, complicated by RHC 80267 itself being an activator of phospholipase C [6].

A stimulatory role for protein kinase C in the response to carbachol is implied by the finding that elevation of intracellular Ca^{2+} by thapsigargin generates a level of secretory activity much below that produced by carbachol, and which is enhanced by the addition of TPA to activate protein kinase C [15]. Furthermore, some of the parietal cell proteins that are phosphorylated in response to carbachol are also phosphorylated in response to activation of protein kinase C by TPA [7]. The simplest interpretation of the above results is that protein kinase C mediates part of the stimulatory action of carbachol through protein phosphorylation.

Recently, a series of highly specific bisindolylmaleimide inhibitors of protein kinase C have been developed [16]. We have used one of these, Ro 31-8220 (compound 3 in Ref. 16), to investigate the role of protein kinase C in the stimulation of acid secretion by carbachol in rat parietal cells. The intracellular accumulation of the weak base

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† Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; IBMX, 3-isobutyl-1-methylxanthine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

aminopyrine [17, 18] was used as an index of secretory activity. The results demonstrate that Ro 31-8220 can be used successfully to investigate the involvement of protein kinase C in a signal transduction pathway involving a muscarinic cholinergic receptor and suggest that it could be used with other cell types to resolve questions concerning the effects of activation of protein kinase C by such receptors [10].

MATERIALS AND METHODS

Preparation and incubation of parietal cells. A crude suspension containing 20–22% parietal cells (mean ± SEM for 10 preparations = 21 ± 1) was prepared from everted sacs of rat corpus by digestion with pronase and intermittent Ca^{2+} chelation as described previously [18]. Parietal cells were enriched by centrifugation on a self-generating Percoll gradient [18]. The low-density cell fraction removed from the top 20% of the gradient always contained >80% parietal cells (mean ± SEM for 10 preparations = 85 ± 1). This fraction was washed free from Percoll by centrifugation at 200 *g* for 5 min at 15°, was resuspended in Eagle's minimum essential medium containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), 1 mg/mL of bovine serum albumin, 8 μg/mL insulin, 10 nM hydrocortisone, 5% foetal calf serum and 50 μg/mL of gentamicin and then incubated with gentle shaking for 2 hr at 37° with continuous gassing with 95% O_2/5% CO_2. This preincubation procedure increased the subsequent response of the enriched cells to secretagogues [18].

Aminopyrine accumulation. Cells were suspended in Eagle's minimum essential medium containing 20 mM HEPES and 1 mg/mL bovine serum albumin to which [^{14}C]aminopyrine (0.1 μCi/mL; 0.9 μM) and [^{3}H]polyethylene glycol (0.4 μCi/mL) had been added, and were incubated for 30 min at 37° in plastic vials that had been gassed with 95% O_2/5% CO_2 and capped. TPA and Ro 31-8220 were dissolved in dimethyl sulphoxide and all other agents were dissolved in saline (NaCl, 9 g/L). The appropriate vehicle was added to control vials, the final concentration of dimethyl sulphoxide being 0.12% (v/v). After 30-min incubation, samples of suspension were centrifuged at 10,000 *g* for 30 sec, the cell pellets dissolved in 1 M sodium hydroxide and the radioactive content estimated by scintillation counting using OptiPhase HiSafe II (Pharmacia). The aminopyrine accumulation ratio was calculated by dividing the intracellular concentration of aminopyrine by that in the medium as described previously [18].

Aminopyrine accumulation only occurs in parietal cells [19], and it is therefore possible to assess acid secretory activity without enrichment of parietal cells. The likelihood of products released from other cell types influencing the secretory activity of parietal cells is small because any such mediators will be substantially diluted in the incubation medium, and enrichment to 80–85% parietal cells does not completely remove this possibility. Furthermore, the enrichment procedure may modify the function of parietal cells. These considerations led us to investigate the effects of Ro 31-8220 by using both unenriched and enriched preparations of parietal cells.

Measurement of intracellular Ca^{2+}. Enriched parietal cells were suspended at 2.5×10^6 cells/mL in Eagle's minimum essential medium containing 20 mM HEPES, 1 mg/mL bovine serum albumin and 2 μM FURA-2 acetoxymethylester, and were incubated for 20 min at 37°. Loading and formation of FURA-2 from the acetoxymethyl ester were monitored by the shift in the cellular fluorescence emission peak from 488 to 502 nm. The cells were washed and resuspended (0.5×10^6 cells/mL) in a balanced salt solution (pH 7.4) [20] consisting of 0.5 mM NaH_2PO_4, 1.0 mM Na_2HPO_4, 20 mM $NaHCO_3$, 70 mM NaCl, 5 mM KCl, 1.0 mM $CaCl_2$, 50 mM HEPES, 10 mM glucose and 0.1 mM isoleucine which had been gassed with 95% O_2/5% CO_2. They were equilibrated in a Perkin–Elmer L5 spectrofluorimeter cuvette with the stirrer set on low for 3 min at 37°. After 30 sec, Ro 31-8220, atropine or vehicle (saline, or dimethyl sulphoxide at 0.08% (v/v) final concentration) was added, followed by carbachol (0.1 mM) after a further 120 sec. The response to carbachol was recorded for 150 sec. Measurement was made of the emission ratio (R) at 510 nm from alternating excitation at 340 and 380 nm, and the intracellular Ca^{2+} concentration was calculated by computer program (Perkin–Elmer) from the equation [21]:

$$[Ca^{2+}] = K_d[(R - R_{min})/(R_{max} - R)]\,(S_{f2}/S_{b2}).$$

Where K_d = 224 nM, R_{min} = emission ratio in the presence of Triton X-100 (0.2%, v/v) and 20 mM EGTA, R_{max} = emission ratio in the presence of Triton X-100 (0.2%, v/v) alone, S_{f2}/S_{b2} = the ratio of the emission intensities of the free and bound probe with excitation at 380 nm.

Materials. Male Wistar rats (200 g) were obtained from Bantin & Kingman (Hull, U.K.) and were fed on Heygates breeding diet supplied by Pilsbury (Birmingham, U.K.). Ro 31-8220 was a generous gift from Dr G. Lawton, Roche Products (Welwyn Garden City, U.K.). Pronase was from BDH (Poole, U.K.) all other reagents were from the Sigma Chemical Co. (Poole, U.K.).

Presentation of results. Results are presented as means ± SEM from N separate cell preparations. Data on aminopyrine accumulation are presented in normalized form to reduce the effect of variation between cell batches [17]. All statistical analyses were performed on data before normalization.

RESULTS

Antagonism by Ro 31-8220 of the inhibitory effect of TPA on histamine-stimulated aminopyrine accumulation

TPA activates protein kinase C in rat parietal cells and inhibits the stimulation of aminopyrine accumulation by histamine [18]. If Ro 31-8220 inhibits protein kinase C in parietal cells then it should prevent the above inhibitory effect of TPA. As previously [18], 100 nM TPA produced a substantial reduction in the stimulation of the aminopyrine accumulation ratio by 0.5 mM histamine

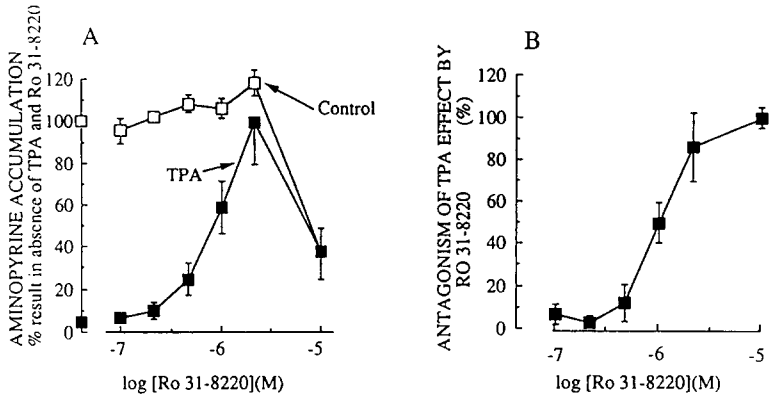

Fig. 1. Antagonism by Ro 31-8220 of the inhibitory effect of TPA on histamine-stimulated aminopyrine accumulation. (A) Results are presented as means ± SEM from four batches of unenriched cells and have been normalized to the stimulation obtained in the presence of 0.5 mM histamine plus 0.1 mM IBMX which was 25 ± 5.4. (□) Control, (■) addition of 100 nM TPA. (B) The antagonism of the effect of TPA by Ro 31-8220 was calculated from data presented in (A) as: [1 − (reduction in aminopyrine accumulation by TPA at a particular concentration of Ro 31-8220/reduction in aminopyrine accumulation by TPA in absence of Ro 31-8220)] × 100. The half-maximally effective concentration of Ro 31-8220 was estimated to be 1.0 μM (computer program FIT [22]).

and 0.1 mM 3-isobutyl-1-methylxanthine (IBMX) (Fig. 1A, compare open and filled points on vertical axis). Ro 31-8220 produced a concentration-dependent reduction in the inhibitory effect of TPA on aminopyrine accumulation (Fig. 1A). The half-maximally effective concentration of Ro 31-8220 was estimated by using the computer program FIT [22] to be 1.0 μM (Fig. 1B).

Effect of Ro 31-8220 *on histamine-stimulated aminopyrine accumulation*

In unenriched cells Ro 31-8220, over the range 0.1–2.14 μM, produced a slight enhancement of the aminopyrine ratio stimulated by 0.5 mM histamine ($P < 0.05$ by analysis of variance). However, increasing the concentration of Ro 31-8220 from 2.14 to 10 μM produced a decrease in the aminopyrine accumulation ratio (Fig. 1A). Ro 31-8220 at 2.14 μM had a significant effect ($P < 0.05$ paired *t*-test) on the dose–response curve for histamine-stimulated aminopyrine accumulation only at the highest concentration of histamine (Fig. 2). Basal aminopyrine accumulation was unaffected by Ro 31-8220 (see legends to Figs 2 and 3). In an enriched preparation of parietal cells 2.14 μM Ro 31-8220 had no effect on histamine-stimulated aminopyrine accumulation (Table 1).

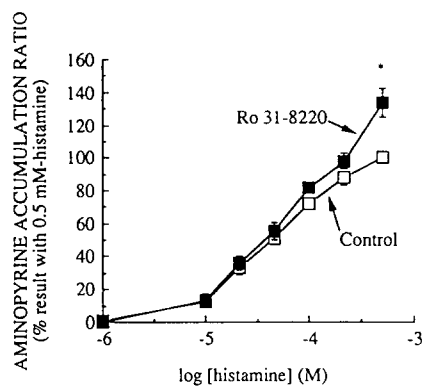

Fig. 2. Effect of Ro 31-8220 on the secretory response of unenriched parietal cells to different concentrations of histamine. Results are from six experiments and are expressed as means ± SEM. (□) Control, (■) 2.14 μM Ro 31-8220. Results have been normalized to the stimulation of the aminopyrine accumulation ratio obtained with 0.5 mM histamine plus 0.1 mM IBMX which was 18.8 ± 2.8. The basal aminopyrine accumulation was unaffected by the addition of 2.14 μM Ro 31-8220 being 1.9 ± 0.18 and 1.8 ± 0.19, respectively, in the absence and presence of Ro 31-8220. *$P < 0.05$ for effect of TPA by paired *t*-test.

Effect of Ro 31-8220 *on carbachol-stimulated aminopyrine accumulation*

The presence of Ro 31-8220 significantly shifted the dose–response curve for the stimulation of aminopyrine accumulation by carbachol downwards and to the right (Fig. 3A, $P < 0.05$ by factorial analysis of variance). The half-maximally effective concentration of Ro 31-8220 for the inhibition of secretory activity stimulated by 0.1 mM carbachol was 0.78 μM (Fig. 3B). A similar inhibitory effect of 2.14 μM Ro 31-8220 on stimulation of aminopyrine accumulation by 0.1 mM carbachol was obtained with enriched parietal cells (Table 1).

Lack of effect of Ro 31-8220 *on the action of carbachol on intracellular* Ca^{2+}

In enriched parietal cells, which had been loaded with FURA-2, 0.1 mM carbachol produced a rapid

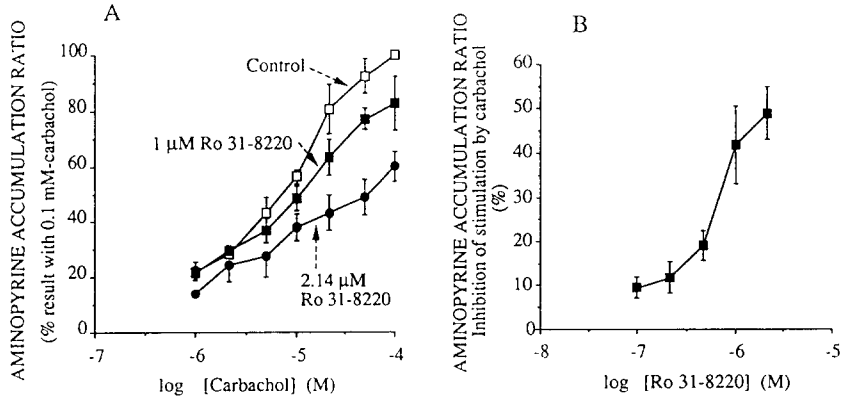

Fig. 3. Effect of Ro 31-8220 on the stimulation of aminopyrine accumulation by carbachol. Results are means ± SEM from four experiments in each case and were performed with a suspension containing unenriched parietal cells. (A) Effect of the concentration of carbachol in the absence (□), and presence of 1 μM (■) and 2.14 μM (●) Ro 31-8220. Results have been normalized to the stimulation of aminopyrine accumulation obtained with 0.1 mM carbachol alone. The basal aminopyrine accumulation ratio was 1.7 ± 0.14 and results in the presence of 1 μM and 2.14 μM Ro 31-8220 were 1.6 ± 0.14 and 1.6 ± 0.14, respectively. Aminopyrine accumulation in the presence of 0.1 mM carbachol was 4.1 ± 0.3. (B) Effect of concentration of Ro 31-8220 on the response to 0.1 mM carbachol. Basal aminopyrine accumulation was 1.8 ± 0.10, and that in the presence of 0.1 mM carbachol was 3.36 ± 0.38.

Table 1. Effect of 2.14 μM Ro 31-8220 on aminopyrine accumulation in a suspension containing greater than 80% parietal cells

Agent	Stimulation of aminopyrine accumulation		Inhibition by Ro 31-8220 (%)
	Control	2.14 μM Ro 31-8220	
0.5 mM Histamine + 0.1 mM IBMX	14.28 ± 1.4	14.7 ± 1.3	−3.06 ± 3.3
0.1 mM Carbachol	2.53 ± 0.26	1.44 ± 0.16*	43.4 ± 3.82

Results are means ± SEM of four experiments. *$P < 0.01$ for effect of Ro 31-8220.
Basal aminopyrine accumulation was 1.9 ± 0.05 and 1.9 ± 0.07 with the addition of 0.1 mM IBMX.

rise in intracellular Ca^{2+} which then fell to a plateau which was significantly ($P < 0.01$) above basal (Fig. 4). This effect of carbachol was completely abolished by the prior addition of 10 μM atropine ($N = 3$) (Fig. 4). No effect of Ro 31-8220 on intracellular Ca^{2+} under basal conditions or on the changes in intracellular Ca^{2+} induced by stimulation with carbachol was detectable (Table 2).

DISCUSSION

Before using Ro 31-8820 to investigate the involvement of protein kinase C in the action of carbachol it was necessary to show that Ro 31-8220 could inhibit protein kinase C in parietal cells. The simplest way to demonstrate this was to use the phorbol ester TPA to activate protein kinase C in these cells. Ro 31-8220 proved an effective antagonist of the previously demonstrated [18] inhibitory effect of TPA on histamine-stimulated secretory activity, and the IC_{50} of 1.0 μM was close to that obtained for the inhibition of effects of TPA by Ro 31-8220 in other cell types [16]. Ro 31-8220 inhibited aminopyrine accumulation in response to histamine only at a concentration of 10 μM. This result might be explained by inhibition of cyclic AMP-dependent protein kinase, on which the action of histamine depends [23], for high concentrations of Ro 31-8220 could potentially inhibit this enzyme [16]. At concentrations of Ro 31-8220 of 2.14 μM and below there was no significant inhibition by this compound of the response to histamine, but antagonism of the effect of TPA was clearly apparent. Ro 31-8220 therefore appeared to exert a selective inhibition of protein kinase C with negligible inhibitory effect on cyclic AMP-dependent protein kinase at concentrations of 2.14 μM and below. These results accord with experiments using isolated preparations of cyclic AMP-dependent protein kinase and protein kinase C where the IC_{50} for inhibition of the former enzyme by Ro 31-8220 was 150 times higher than that for protein kinase C [16].

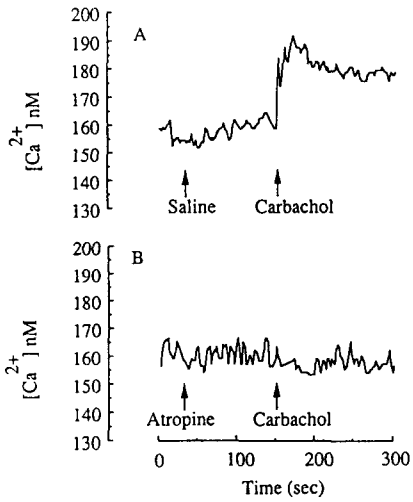

Fig. 4. Effect of 0.1 mM carbachol on intracellular Ca^{2+} in a suspension containing >80% parietal cells was prevented by prior addition of 0.01 mM atropine. For details see Materials and Methods.

Ro 31-8220 inhibited the stimulatory effect of carbachol. The evidence that this effect of Ro 31-8220 involved inhibition of protein kinase C is now discussed. Firstly, the IC_{50} for the inhibition of the response to carbachol by Ro 31-8220 was similar to that for antagonism of the effects of the activator of protein kinase C, TPA, in parietal and other [16] cells. The response to carbachol is dependent on the rise in intracellular Ca^{2+} [7, 9] and it was therefore necessary to exclude the possibility that Ro 31-8220 was acting on intracellular Ca^{2+} rather than protein kinase C. Changes in intracellular Ca^{2+} were monitored by loading the parietal cells with FURA-2 and monitoring the emission ratio at 510 nm from excitation at 340 and 380 nm. The intracellular calcium concentration was calculated from this ratio by using the equation derived by Grynkiewicz *et al.* [21]. This equation does not take account of the effect of cytosolic viscosity on the fluorescence of FURA-2 [24] and intracellular Ca^{2+} may therefore be consistently underestimated. Despite this reservation the conclusions that the action of carbachol on intracellular Ca^{2+} was mediated via the muscarinic receptor, because it was prevented by 0.01 mM atropine, and that Ro 31-8220 did not affect the change in Ca^{2+} produced by carbachol remain valid. Ro 31-8220 must therefore have been exerting its inhibitory effect on a signalling pathway activated by carbachol other than elevation of intracellular Ca^{2+}. The most likely pathway is that involving protein kinase C. As with other preparations of parietal cells [25, 26] the aminopyrine response to carbachol was less than the response to histamine plus IBMX. However, examination of the effect of 2.14 μM Ro 31-8220 on the dose–response curve to histamine demonstrates that this compound did not inhibit the response to histamine even when the level of secretory activity was similar to that induced by carbachol. Therefore, a final point is that non-specific effects of Ro 31-8220 on aminopyrine accumulation, or on the acid secretory process, can be excluded as an explanation of the effects of this agent on carbachol-induced secretory activity.

In unenriched cells 2.14 μM Ro 31-8220 stimulated the response to 0.5 mM histamine. This result could represent antagonism of an inhibitory modulation of the histamine response by a basal protein kinase C activity [18], and its absence from enriched cells could reflect changes in basal protein kinase C activity during the enrichment procedure. More experiments are required to clarify these effects, but such information is not essential to the interpretation of the current study on the involvement of protein kinase C in the response of parietal cells to carbachol.

The present findings, along with results on the enhancement of the response to thapsigargin by TPA [15], suggest that the predominant role for protein kinase C is in activation of the secretory response to carbachol. Extrapolation of the dose–response curve for Ro 31-8220 shows that even at a maximally effective concentration of the inhibitor the response to carbachol would not be abolished. Therefore, protein kinase C may play an important facilitatory role, rather than an essential one. The inhibitory effect of TPA on the response to carbachol [4, 12, 13] may be explained by this agent producing a pharmacological and irreversible activation of protein kinase C which is different from that

Table 2. Lack of effect of Ro 31-8220 on intracellular Ca^{2+} in a suspension enriched with rat parietal cells

	Intracellular Ca^{2+} (nM)			
Treatment	0–30 sec	31–150 sec	At peak response	At plateau (240–300 sec)
Control	187 ± 32	194 ± 33	238 ± 40	216 ± 36
Ro 31-8220	173 ± 31	181 ± 32	227 ± 42	205 ± 35

Results are means ± SEM of data from seven separate preparations enriched with parietal cells. Ro 31-8220 (2.14 μM) or vehicle was added at 30 sec and carbachol (0.1 mM) at 150 sec. The peak response was the highest single value for intracellular Ca^{2+} after the addition of carbachol; other results are the average values over the time periods specified. Results were compared by a paired *t*-test.

produced by carbachol. However, protein kinase C may genuinely be involved in feedback regulation of the response to carbachol but this action may be of less importance than the stimulatory role of protein kinase C during the 30-min incubation used in the present experiments. Ro 31-8220 is a competitive antagonist for the ATP binding site on protein kinase C and since this region is conserved in all isoforms [10] it seems unlikely that Ro 31-8220 will show substantial isoform selectivity. Different isoforms of protein kinase C could however be involved in the multiplicity of actions of protein kinase C in parietal cells [10, 27].

In conclusion, the present work demonstrates that Ro 31-8220 can be successfully used to elucidate the involvement of protein kinase C in a signal transduction pathway involving receptor-mediated breakdown of phosphatidylinositol 4,5-bisphosphate, and shows that in parietal cells the predominant role for protein kinase C in the response to carbachol is a stimulatory one.

Acknowledgement—This work was supported by MRC, U.K.

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