Contrasting effects of phorbol ester and agonist-mediated activation of protein kinase C on phosphoinositide and Ca^{2+} signalling in a human neuroblastoma

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The effects of protein kinase C (PKC) activation on muscarinic receptor-mediated phosphoinositide and Ca^{2+} signalling were examined in the human neuroblastoma, SH-SY5Y. Carbachol evoked rapid transient elevations of $\text{Ins}(1,4,5)\text{P}_3$ and intracellular $[\text{Ca}^{2+}]$ followed by lower sustained elevations. Phorbol 12,13-dibutyrate (PDBu) preferentially attenuated transient phases. Removal of the transplasmalemmal Ca^{2+} gradient coupled with depletion of intracellular Ca^{2+} stores with thapsigargin also reduced carbachol-mediated $\text{Ins}(1,4,5)\text{P}_3$ accumulation. Under these conditions, PDBu virtually abolished $\text{Ins}(1,4,5)\text{P}_3$ responses to carbachol thereby implicating both Ca^{2+} - and PKC-sensitive components. PDBu also reduced agonist-mediated accumulation of inositol phosphates and depletion of lipids, thereby eliminating an effect of PKC on $\text{Ins}(1,4,5)\text{P}_3$ metabolism or phosphoinositide synthesis. In electroporated cells, PDBu inhibited $\text{Ins}(1,4,5)\text{P}_3$ accumulation mediated by carbachol or guanosine 5'-[γ -thio]-triphosphate, the latter indicating that some PDBu-sensitive

elements were downstream of the receptor. The PKC inhibitor, Ro-318220, protected against PDBu but did not enhance responses to maximal concentrations of carbachol, indicating no feedback inhibition by agonist-activated PKC. Muscarinic antagonist activity of Ro-318220 complicated such assessment at low agonist concentrations. Carbachol or PDBu induced cytosol to membrane translocation of $\text{PKC}\alpha$. This was faster and possibly greater with PDBu, which may explain the lack of feedback by agonist-activated PKC. These results indicate that, in SH-SY5Y cells, PDBu activation of PKC preferentially inhibits rapid muscarinic receptor-mediated phosphoinositide and Ca^{2+} responses via suppression of $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis. This is at least partially through inhibition of G_q -protein/phosphoinositidase C coupling. However, at least at high agonist concentrations, a major agonist-mediated PKC feedback is not present in these cells.

INTRODUCTION

Agonist occupation of a wide variety of cell-surface receptors increases the activity of at least one isoform of phosphoinositidase C (PIC) to enhance the hydrolysis of the minor membrane phospholipid, $\text{PtdIns}(4,5)\text{P}_2$. This hydrolysis generates both $\text{Ins}(1,4,5)\text{P}_3$ and 1,2-diacylglycerol which are able to mobilize Ca^{2+} from intracellular stores and activate protein kinase C (PKC) respectively [1]. The sensitivity and responsiveness of cells to activation of cell-surface receptors may be influenced by the internalization and degradation of such receptors. Other components of this pathway may be similarly regulated, such that down-regulation of both G-proteins [2] and the $\text{Ins}(1,4,5)\text{P}_3$ receptor [3] may occur following exposure to agonists for many hours. These processes may mediate cellular desensitization to relatively long periods of agonist exposure.

The phosphoinositide pathway is also subject to acute regulation, a striking example of which is the rapid but often partial desensitization of PIC activity that occurs within seconds of agonist occupation of muscarinic [4–6], and other receptor types [7,8]. Additionally, features may exist which have the potential to influence the magnitude of cellular responses and their sensitivity to agonist. One such feature may result from the activation of PKC(s) and indeed this has been implicated in the limitation of muscarinic receptor-mediated activation of PIC [9–14] and intracellular $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_i$) elevation [10,15]. Although such

studies demonstrate the potential for PKC-mediated inhibition of phosphoinositide responses by the use of phorbol esters, few have explored the role of muscarinic receptor-mediated PKC feedback regulation and the site(s) of PKC action. Furthermore, PIC activity has generally been assessed by measurement of the accumulation of total [^3H]inositol phosphates ($[\text{P}^3\text{H}]\text{InsP}_x$) in Li^+ -blocked cells over periods of many minutes. While this technique provides an index of total PIC activity, the rapid desensitization of muscarinic receptor-mediated activation of PIC means that PIC activity is not constant over such time periods. Thus, accumulation of $[\text{P}^3\text{H}]\text{InsP}_x$ over many minutes will provide information about a partially desensitized receptor. Regulatory features under such circumstances may differ from those of a fully sensitized receptor [16].

In this study we have examined the potential regulatory influence of PKC, activated by either phorbol ester or agonist occupation of muscarinic receptors (predominantly M_3 [17]), on phosphoinositide and $[\text{Ca}^{2+}]_i$ signalling in the human neuroblastoma cell line, SH-SY5Y.

MATERIALS AND METHODS

Cell culture

Experiments were performed on SH-SY5Y cells of passages 70–90. Cell culture was carried out as previously described [16]

Abbreviations used: ANOVA, analysis of variance; $[\text{Ca}^{2+}]_o$, extracellular $[\text{Ca}^{2+}]$; $[\text{Ca}^{2+}]_i$, intracellular $[\text{Ca}^{2+}]$; CHO, Chinese hamster ovary; ECL, enhanced chemiluminescence; [^3H]GroPIns, [^3H]glycerophosphoinositol; [^3H]GroPIns(4) P , [^3H]glycerophosphoinositol 4-phosphate; [^3H]GroPIns(4,5) P_2 , [^3H]glycerophosphoinositol 4,5-bisphosphate; GTP[S], guanosine 5'-[γ -thio]triphosphate; [^3H]Ins P_x , total [^3H]inositol phosphates; ICB, intracellular-like buffer; [^3H]NMS, 1-[N-methyl- ^3H]scopolamine methyl chloride; PDBu, phorbol 12,13-dibutyrate; PIC, phosphoinositidase C; PKC, protein kinase C; TCA, trichloroacetic acid.

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and cells harvested with 10 mM Hepes containing 154 mM NaCl and 0.54 mM EDTA (pH 7.4).

Generation of $\text{Ins}(1,4,5)\text{P}_3$ in intact cells

Cells were harvested and resuspended in Krebs/Hepes buffer [pH 7.4, composition (mM): Hepes 10, NaHCO_3 4.2, glucose 11.7, MgSO_4 1.2, KH_2PO_4 1.2, KCl 4.7, NaCl 118 and CaCl_2 1.3] at 37 °C. For $\text{Ins}(1,4,5)\text{P}_3$ generation at reduced extracellular $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_e$), cells were washed in the appropriate buffer. In these experiments and those described below, the $[\text{Ca}^{2+}]$, determined with fura-2 [18], was either 2–10 μM in nominally Ca^{2+} -free conditions or buffered to 50–100 nM with EGTA. Where required the PKC inhibitor, Ro-318220, was added 10 min before and phorbol 12,13-dibutyrate (PDBu) 5 min before carbachol. Cells (90 μl , 100–300 μg of protein) were added to 10 μl of agonist at 37 °C and reactions terminated with an equal volume of ice-cold 1 M trichloroacetic acid (TCA). $\text{Ins}(1,4,5)\text{P}_3$ was extracted and measured by specific radioreceptor assay as described elsewhere [16] and referenced to total cellular protein determined [19] in 0.1 M NaOH digests using bovine albumin fraction V as standard.

Cells were treated for 24 h with phorbol ester to down-regulate PKC by addition of 1 μM PDBu to confluent cell monolayers in 24-well plates (approx. 200 μg of protein/well). Vehicle (DMSO 0.1 %, v/v) was added to control cultures. After 24 h, cells were washed and equilibrated in Krebs/Hepes buffer (1 ml) for 30 min at 37 °C. Buffer was replaced with 100 μl of agonist. Where required, PDBu or vehicle were added 5 min prior to agonist. Reactions were terminated with an equal volume of ice-cold 1 M TCA and processed as above.

Generation of $\text{Ins}(1,4,5)\text{P}_3$ in permeabilized cells

Cells were harvested and resuspended in 1.6 ml of an intracellular-like buffer (ICB) [pH 6.9, composition (mM): Hepes 20, KCl 10, $(\text{NaH}_2\text{COONa})_2$ 5, MgCl_2 2.4, KH_2PO_4 2, ATP 2]. The free $[\text{Ca}^{2+}]$ of this buffer was set to approx. 300 nM with EGTA as both carbachol- and guanosine 5'-[γ -thio]triphosphate (GTP[S])-stimulated $\text{Ins}(1,4,5)\text{P}_3$ responses are Ca^{2+} -sensitive and maximal at this concentration [5]. A 0.8 ml aliquot of cells was treated with 1 μM PDBu or 10 μM Ro-318220 or vehicle (DMSO, 0.1 % v/v) for 5 min at 37 °C. After 3 min on ice, cells were electroporated with three discharges, separated by 20 s, of a 3 μF capacitor with a field strength of 3.75 kV/cm and a time constant of 0.1 ms. An equal volume of ICB (37 °C with appropriate test agent) was then added and cells incubated for 5 min at 37 °C. Generation and measurement of $\text{Ins}(1,4,5)\text{P}_3$ on cells in suspension were then performed as above, with 90 μl of cells containing 200–300 μg of protein.

Determination of $[\text{Ca}^{2+}]_i$

Harvested cells (approx. 4–6 mg of protein) were resuspended in Krebs/Hepes buffer and loaded with fura-2 acetoxymethyl ester (5 μM) for 40–50 min at room temperature. Aliquots of cells (0.5–1.5 mg of protein) were harvested (800 g, 20 s) and resuspended in cuvettes in 3 ml of Krebs/Hepes buffer. Cells were maintained at 37 °C from this point. For studies at reduced $[\text{Ca}^{2+}]_e$, cells were washed with 1 ml of the appropriate buffer prior to final suspension. Either 1 μM PDBu or vehicle were added 5 min prior to recording while Ro-318220 was added 10 min before measurement. With emission at 509 nm, the 340 nm/380 nm ratio (each 3.8 s) was recorded as an index of $[\text{Ca}^{2+}]_i$. R_{max} was determined with 0.1 % Triton X-100 (and Ca^{2+} if required) and R_{min} with 6.7 mM EGTA. Following subtraction

of autofluorescence, these values were used to convert 340 nm/380 nm ratios into $[\text{Ca}^{2+}]_i$ [18].

Measurement of $[\text{H}^3]\text{InsP}_x$ accumulation

This was as previously described [16] with the exception that preincubation with 5 mM Li^+ was for 5 min. Cells were treated during this period with either 1 μM PDBu or vehicle and agonist added in buffer containing either PDBu or vehicle.

Measurement of $[\text{H}^3]$ phosphoinositides and $[\text{H}^3]\text{InsP}_x$ in the absence of Li^+

For the determination of the glycerophosphoinositols, $[\text{H}^3]\text{GroPIns}$, $[\text{H}^3]\text{GroPIns}(4)\text{P}$ and $[\text{H}^3]\text{GroPIns}(4,5)\text{P}_2$ as indices of PtdIns, PtdIns(4)P and PtdIns(4,5) P_2 respectively, cells were prelabelled with *myo*- $[\text{H}^3]$ inositol [16]. Cells, grown in 24 well plates, were then preincubated at 37 °C for 30 min in 1 ml of Krebs/Hepes containing 10 mM *myo*-inositol to mask recycling of $[\text{H}^3]$ inositol. PDBu (1 μM) or vehicle was added for the last 5 min of this period. Buffer was then removed and 200 μl of buffer at 37 °C containing agonist and either PDBu or vehicle added. Reactions were stopped with an equal volume of ice-cold 1 M TCA. After 15 min on ice the aqueous phase was removed and processed [16] to allow determination of $[\text{H}^3]\text{InsP}_x$. Lipids were extracted into chloroform/methanol/ 10 M hydrochloric acid (40:80:1, by vol.) and following phase partition a volume of the chloroform phase was taken and left to dry. Phospholipids were deacylated by mild alkaline hydrolysis and the resultant water-soluble glycerophosphoinositols separated by anion-exchange chromatography [20].

1-[*N*-methyl- ^3H]Scopolamine methyl chloride ($[\text{H}^3]\text{NMS}$) binding

The influence of activators and inhibitors of PKC on the binding of the muscarinic antagonist, $[\text{H}^3]\text{NMS}$ was assessed using recombinant human muscarinic M_3 receptors expressed in Chinese hamster ovary (CHO) cells. This model was used as the high level of receptor expression (1 pmol/mg of protein [21]) would allow accurate determination of any $[\text{H}^3]\text{NMS}$ displacement when used around its K_d of approx. 0.3 nM [21]. Cells in 24-well multiwells (20–30 % confluent, 30 μg of protein/well) were washed and incubated for 1 h at 37 °C (in the light) in 1 ml of Krebs/Hepes with $[\text{H}^3]\text{NMS}$ and the test agent. Non-specific binding was determined with 10 μM atropine. Cells were washed rapidly with two 1 ml volumes of ice-cold buffer and digested with 0.5 ml of 0.1 M NaOH. This was neutralized with 0.5 ml of 1 M TCA and ^3H determined by liquid scintillation spectrometry in 5 ml of Floscent IV.

Preparation of cell extracts and immunoblot analysis of PKC isoenzymes

Western blots indicate that PKC α and PKC β 1 are the predominant PKC isoforms in SH-SY5Y cells [22]. As an index of PKC activation, the translocation of PKC α from the cytosolic to membrane fraction was therefore determined following carbachol or PDBu treatment.

Harvested cells were washed twice and suspended in Krebs/Hepes buffer. Cells (300 μl , approx. 1.5 mg of protein) were incubated with 1 mM carbachol or 1 μM PDBu at 37 °C for the indicated times. Cells were pelleted (10000 g, 3 min) and lysed in ice-cold homogenization buffer (20 mM Tris/HCl, 1 mM dithiothreitol, 5 mM EGTA, 2 mM EDTA, 0.5 mM PMSF, 1 μg /ml aprotinin, 10 μM benzamidine hydrochloride, 5 μM iodo-

acetamide, pH 7.4) using 10 passes of a motor-driven glass-Teflon homogenizer. Homogenates were centrifuged (33000 *g*, 1 h, 4 °C) to yield supernatants (cytosolic extracts) and pellets (membrane fractions). Samples were denatured by boiling in an equal volume of sample buffer [50 mM Tris, 2% SDS, 0.1% Bromophenol Blue, 10% (v/v) glycerol, 5% (v/v) mercaptoethanol] and subjected to SDS/PAGE using a 10% running gel. Proteins were transferred to nitrocellulose which was blocked (20 °C, 1 h) with 5% (w/v) dry milk in TTBS (20 mM Tris/HCl, 0.15 M NaCl, 0.05% Tween-20, pH 7.5). Mouse anti-PKC α antibodies (1:3000 in TTBS, 1% dry milk) were then added for 2 h followed by horseradish peroxidase-labelled anti-(mouse IgG) antibody for 1 h. Nitrocellulose was washed following both incubations with TTBS (2 $\times$ 1 min, 1 $\times$ 15 min, 2 $\times$ 5 min). Immunoreactive bands were detected with enhanced chemiluminescence (ECL) reagents and exposure to Hyperfilm-ECL.

Statistics and data presentation

Inhibition curves and IC_{50} values were determined with GraphPAD (Academic Press Inc. 1986) using a standard four-parameter logistic equation. Where full time courses of carbachol-mediated responses were compared with and without treatment with PDBu, analysis was by two-way analysis of variance (ANOVA). Comparisons between individual group means was by two-tailed, unpaired, Student's *t*-test. Multiple comparisons between group means were by Duncan's multiple-range test at $P < 0.05$ and $P < 0.01$ following one-way ANOVA with $P < 0.05$. For clarity all data are presented as means $\pm$ S.E.M.

Materials

Reagents of analytical grade were obtained from the suppliers listed previously [16,20,23] or from Sigma (Poole, U.K.). In addition, PDBu, calphostin-C and chelerythrine chloride were from Calbiochem (Nottingham, U.K.). Ro-318220 was a gift from Dr. G. Lawton, Roche Products Ltd. (Welwyn Garden City, Herts., U.K.). Hyperfilm, ECL reagents for Western blotting and [^3H]NMS were from Amersham International (Little Chalfont, Bucks., U.K.). Mouse anti-PKC α antibody and horseradish peroxidase-labelled anti-(mouse IgG) antibody were from Affiniti Research Products Ltd. (Nottingham, U.K.) and Sigma (Poole, U.K.) respectively.

RESULTS AND DISCUSSION

This study examined the effects of phorbol ester activation of PKC on the accumulation of $\text{Ins}(1,4,5)\text{P}_3$ and the elevation of $[\text{Ca}^{2+}]_i$ in SH-SY5Y cells. The potential effects of altered metabolism of $\text{Ins}(1,4,5)\text{P}_3$ on signalling patterns were assessed by measurement of agonist-mediated accumulation of total [^3H]Ins P_x and changes in (poly)phosphoinositides. To investigate the site of PKC action, the effects of phorbol ester on $\text{Ins}(1,4,5)\text{P}_3$ accumulation mediated by direct G-protein activation with GTP[S] in permeabilized cells were determined. The PKC inhibitor, Ro-318220, and down-regulation of PKC by long-term phorbol ester treatment were used to characterize the effects on phosphoinositide signalling of agonist-mediated, as opposed to phorbol ester-mediated, PKC activation.

PDBu attenuates carbachol-mediated $\text{Ins}(1,4,5)\text{P}_3$ and $[\text{Ca}^{2+}]_i$ signalling

Maximal concentrations of carbachol (1 mM) produced rapid but transient peaks of $\text{Ins}(1,4,5)\text{P}_3$ accumulation which subsided over the following minute to lower but sustained levels (Figure

Figure 1 PDBu attenuates carbachol-mediated elevations of $\text{Ins}(1,4,5)\text{P}_3$ and $[\text{Ca}^{2+}]_i$ in SH-SY5Y cells

Time course of changes in $\text{Ins}(1,4,5)\text{P}_3$ mass (**A**) and $[\text{Ca}^{2+}]_i$ (**B**) following challenge with 1 mM carbachol with ($\square$) or without ($\blacksquare$) 5 min pretreatment with 1 μM PDBu. Data are means $\pm$ S.E.M., $n = 3$ or representative of $n = 3$.

1A) confirming the biphasic nature of this response [5,16,24]. Pretreatment of cells for 5 min with 1 μM PDBu and its presence throughout the carbachol challenge significantly ($P < 0.05$) reduced both peak and sustained accumulations (Figure 1A). The greatest effect was on the transient peak, which was reduced by 53% compared with a reduction of 20% during the sustained phase (5 min after carbachol addition). This emphasizes that regulatory influences may differ during different phases of receptor activation [16] and, although the basis of this is unclear, differences would be missed by measuring [^3H]Ins P_x accumulation over many minutes.

The $\text{Ins}(1,4,5)\text{P}_3$ response to carbachol was paralleled by changes in $[\text{Ca}^{2+}]_i$. Thus, there was a rapid peak elevation of $[\text{Ca}^{2+}]_i$ which subsided to a lower but sustained level within 1 min (Figure 1B). PDBu attenuated the peak $[\text{Ca}^{2+}]_i$ response (change of 424 ± 33 nM versus 590 ± 31 nM, $n = 3$, $P < 0.005$) but had no effect on the sustained component (change of 109 ± 3 nM

Figure 2 PDBu or carbachol promoted cytosolic to particulate translocation of PKC α immunoreactivity

Western blots of SH-SY5Y cytosolic and particulate proteins probed with an anti-PKC α antibody demonstrated that treatment with either 1 μ M PDBu or 1 mM carbachol resulted in the loss of cytosolic and appearance of particulate-associated PKC α immunoreactivity. Blots are representative of two showing similar results.

versus 103 ± 7 nM, $n = 3$) confirming a preferential effect on peak responses (see Figure 1B for example).

Translocation of PKC α immunoreactivity

Western blot analysis demonstrated that either carbachol (1 mM) or PDBu (1 μ M) provoked translocation of PKC α from the cytosolic to particulate fraction (Figure 2). Translocation mediated by PDBu occurred within 1 min whereas there was a 1–2 min lag before detectable carbachol-mediated translocation. Immunoreactivity associated with the particulate fraction also suggested that PDBu may produce a greater translocation of PKC α than carbachol.

Extracellular Ca^{2+} requirements of carbachol-mediated $\text{Ins}(1,4,5)\text{P}_3$ and $[\text{Ca}^{2+}]_i$ elevations and its influence on PDBu-induced inhibition

Peak $\text{Ins}(1,4,5)\text{P}_3$ responses to carbachol were similar irrespective of the $[\text{Ca}^{2+}]_e$ (Figures 1A and 3). PDBu also significantly ($P < 0.05$) reduced the peak $\text{Ins}(1,4,5)\text{P}_3$ response to carbachol under nominally Ca^{2+} -free conditions (58%) and at 50–100 nM $[\text{Ca}^{2+}]_e$ (reductions of 58% and 56% respectively). Reducing the $[\text{Ca}^{2+}]_e$ markedly reduced the sustained phase of $\text{Ins}(1,4,5)\text{P}_3$ accumulation (1.3 mM $[\text{Ca}^{2+}]_e$, 67.7 ± 4.8 ; nominally Ca^{2+} -free, 36.6 ± 3.6 ; 50–100 nM $[\text{Ca}^{2+}]_e$, 28.6 ± 6.2 all expressed as pmol/mg of protein, $n = 3$) (Figures 1A and 3). This confirms the facilitatory effect of $[\text{Ca}^{2+}]_e$ on this sustained PIC activity [5,16]

Figure 3 Reducing the $[\text{Ca}^{2+}]_e$ did not affect the ability of PDBu to inhibit carbachol-mediated $\text{Ins}(1,4,5)\text{P}_3$ responses

Time course of changes in $\text{Ins}(1,4,5)\text{P}_3$ mass following challenge with 1 mM carbachol with ($\square$) or without ($\blacksquare$) 5 min pretreatment with 1 μ M PDBu. The $[\text{Ca}^{2+}]_e$ was either 2–10 μ M (A) or 50–100 nM (B). Data are means $\pm$ S.E.M., $n = 3$.

but indicates that it is not obligatory. PDBu inhibited sustained responses at reduced $[\text{Ca}^{2+}]_e$ by a similar proportion to that at 1.3 mM $[\text{Ca}^{2+}]_e$ (approx. 20%) but these were not statistically different.

Compared with the response at 1.3 mM $[\text{Ca}^{2+}]_e$ (103 ± 4 nM, $n = 3$) the sustained $[\text{Ca}^{2+}]_i$ response to carbachol was markedly attenuated in nominally Ca^{2+} -free buffer (25 ± 3 nM, $n = 3$) and abolished at 50–100 nM $[\text{Ca}^{2+}]_e$ (3 ± 1 nM, $n = 3$). This indicates an absolute requirement for $[\text{Ca}^{2+}]_e$. In nominally Ca^{2+} -free conditions the change in $[\text{Ca}^{2+}]_i$ at the peak response to carbachol (508 ± 15 nM, $n = 3$) was similar to that at 1.3 mM $[\text{Ca}^{2+}]_e$ (590 ± 18 , $n = 3$) but at 50–100 nM $[\text{Ca}^{2+}]_e$ the response was substantially reduced (241 ± 11 nM, $n = 3$). The inhibitory effect of PDBu on the peak $[\text{Ca}^{2+}]_i$ response, although observed at 1.3 mM $[\text{Ca}^{2+}]_e$ (see above) and under nominally Ca^{2+} -free conditions (231 ± 22 nM versus 508 ± 15 nM, $n = 3$, $P < 0.0005$), was not observed at 50–100 nM $[\text{Ca}^{2+}]_e$ (201 ± 29 nM versus 241 ± 11 nM, $n = 3$). The reason for this lack of effect of PDBu is unclear. However, under these conditions peak $[\text{Ca}^{2+}]_i$ responses were reduced possibly due to either the loss of cellular Ca^{2+} upon stimulation and/or depletion of Ca^{2+} stores in close proximity to the plasma membrane prior to stimulation.

Table 1 Prevention of the carbachol-mediated elevation of $[\text{Ca}^{2+}]_i$ did not prevent the inhibitory effect of PDBu on the rapid $\text{Ins}(1,4,5)\text{P}_3$ response

$\text{Ins}(1,4,5)\text{P}_3$ responses to 1 mM carbachol with $[\text{Ca}^{2+}]_e$ buffered to 50–100 nM in control (untreated) cells and cells treated prior to agonist addition with 1 μM PDBu for 5 min, 2 μM thapsigargin for 5 min or the two in combination. Data are $\text{Ins}(1,4,5)\text{P}_3$ levels in pmol/mg of protein, mean $\pm$ S.E.M., $n = 3$, $P < 0.01$ for a versus b,c,d and b versus c,d (by Duncan's multiple range test following one-way ANOVA with $P = 0.0001$).

	Control	PDBu	Thapsigargin	Thapsigargin + PDBu
Basal	2.9 ± 2.7	7.2 ± 6.9	11.4 ± 9.8	4.8 ± 4.8
Peak (10 s)	178.5 ± 11.0^a	92.1 ± 8.9^c	80.6 ± 9.3^d	15.0 ± 1.3^b
Plateau (300 s)	27.6 ± 4.8	11.3 ± 2.7	16.2 ± 4.6	9.7 ± 3.0

The primary effect of PDBu was on $\text{Ins}(1,4,5)\text{P}_3$ accumulation and not $[\text{Ca}^{2+}]_i$ signalling

The attenuation by PDBu of peak $\text{Ins}(1,4,5)\text{P}_3$ accumulation but not $[\text{Ca}^{2+}]_i$ elevation at 50–100 nM $[\text{Ca}^{2+}]_e$ in response to carbachol (see above) suggests that the primary effect of PDBu is on $\text{Ins}(1,4,5)\text{P}_3$ generation and not the $[\text{Ca}^{2+}]_i$ response. This is an important consideration given the possible facilitation of PIC activity by Ca^{2+} [5,23,25] and the potential reduction in such facilitation by an effect of PKC on the mobilization, re-uptake or extrusion of Ca^{2+} . This aspect was further investigated by altering the ability of carbachol to elevate $[\text{Ca}^{2+}]_i$.

Carbachol-mediated alterations in $[\text{Ca}^{2+}]_i$ were abolished when cells were challenged in the absence of a transplasmalemmal Ca^{2+} gradient (50–100 nM $[\text{Ca}^{2+}]_e$) following pretreatment for 5 min with thapsigargin (2 μM) to deplete intracellular Ca^{2+} pools (results not shown). Furthermore, peak $\text{Ins}(1,4,5)\text{P}_3$ accumulation was significantly ($P < 0.01$) reduced compared with cells challenged at 50–100 nM $[\text{Ca}^{2+}]_e$ which had not been treated with thapsigargin (Table 1) and which retained a peak $[\text{Ca}^{2+}]_i$ response (see above). This confirms the suggestion that Ca^{2+} mobilized from intracellular stores is able to facilitate PIC activity [5,23]. Combined treatment with PDBu and thapsigargin virtually abolished the carbachol-mediated peak $\text{Ins}(1,4,5)\text{P}_3$ accumulation when cells were challenged at 50–100 nM $[\text{Ca}^{2+}]_e$ (Table 1). This indicates that PDBu is able to influence the $\text{Ins}(1,4,5)\text{P}_3$ response independently of an effect on $[\text{Ca}^{2+}]_i$ and furthermore suggests that $\text{Ins}(1,4,5)\text{P}_3$ accumulation consists of a $[\text{Ca}^{2+}]_i$ -sensitive and a PKC-sensitive component. Whether these sensitivities reside at identical or different sites (e.g. identical or different PIC isoforms) is unclear. There was a minor sustained accumulation of $\text{Ins}(1,4,5)\text{P}_3$ in response to carbachol at 50–100 nM $[\text{Ca}^{2+}]_e$ and this was not significantly affected by treatment with PDBu or thapsigargin alone or in combination (Table 1).

PDBu attenuated carbachol-mediated $\text{Ins}(1,4,5)\text{P}_3$ accumulation by inhibiting hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$

The accumulation of either $\text{Ins}(1,4,5)\text{P}_3$ or $[\text{H}]\text{InsP}_x$ is indicative of the net balance between both generation and metabolism, and net accumulation may be influenced by changes in one or both. However, in cells in which inositol monophosphatase activity has been blocked with Li^+ , the accumulation of $[\text{H}]\text{InsP}_x$ [but not $\text{Ins}(1,4,5)\text{P}_3$] will be independent of metabolism and indicate total PIC activity. In cells equilibrium-labelled with $[\text{H}]\text{inositol}$ carbachol produced a biphasic, time-dependent accumulation of $[\text{H}]\text{InsP}_x$ in the presence of Li^+ (Figure 4A). There was an initial rapid accumulation over the first 1 min followed by a slower but

sustained accumulation. This biphasic accumulation suggests that the biphasic $\text{Ins}(1,4,5)\text{P}_3$ response may reflect the rapid partial desensitization of PIC as discussed elsewhere [4]. PDBu caused a significant ($P = 0.0009$) inhibition of $[\text{H}]\text{InsP}_x$ accumulation over the course of the experiment (Figure 4A). This indicates that the effect of PDBu is not solely via an effect upon the metabolism of $\text{Ins}(1,4,5)\text{P}_3$ and results in an inhibition of PIC activity.

PDBu addition to $[\text{H}]\text{inositol}$ -labelled cells, in the absence of Li^+ or agonist (but with 10 mM inositol to ensure no recycling of $[\text{H}]\text{inositol}$) did not affect the absolute or relative levels of $[\text{H}]\text{PtdIns}$, $[\text{H}]\text{PtdIns}(4)\text{P}$ and $[\text{H}]\text{PtdIns}(4,5)\text{P}_2$ the latter comprising only approx. 3% of the total (poly)phosphoinositide pool. Thus, despite possible regulatory effects of PKCs on inositol phospholipid kinases [26] and inositol phospholipid movements between different cellular compartments [27], no effects of PDBu were observed in the absence of muscarinic receptor activation.

Under these conditions, carbachol produced a rapid accumulation of $[\text{H}]\text{InsP}_x$ over the first 60 s followed by a sustained elevation (Figure 4B). PDBu significantly ($P = 0.012$) inhibited this accumulation. In these cells, carbachol also rapidly decreased levels of $[\text{H}]\text{PtdIns}(4,5)\text{P}_2$ (Figure 4C) and $[\text{H}]\text{PtdIns}(4)\text{P}$ (Figure 4D) by 76% and 70% respectively which then recovered slightly. In contrast, the level of $[\text{H}]\text{PtdIns}$ fell by only approx. 10% over the first 60 s of stimulation (990407 ± 40525 d.p.m. to 899981 ± 45364 d.p.m., $n = 4$) but continued to fall to $85.8 \pm 4.2\%$ of basal levels after 15 min of stimulation. PDBu significantly inhibited the degree of depletion of both $[\text{H}]\text{PtdIns}(4,5)\text{P}_2$ ($P = 1.38 \times 10^{-6}$) and $[\text{H}]\text{PtdIns}(4)\text{P}$ ($P = 2.21 \times 10^{-6}$) (Figures 4C and 4D) but did not influence the depletion of $[\text{H}]\text{PtdIns}$ (results not shown).

The extent of carbachol-mediated depletion of $[\text{H}]\text{PtdIns}(4)\text{P}$ and $[\text{H}]\text{PtdIns}(4,5)\text{P}_2$ were similar to those reported recently for muscarinic receptor stimulation of this cell line [6] and other cell types including CHO cells expressing recombinant human muscarinic M_1 receptors [20]. Given the relatively small depletion of $[\text{H}]\text{PtdIns}$ the specific activities of $[\text{H}]\text{PtdIns}(4)\text{P}$ and $[\text{H}]\text{PtdIns}(4,5)\text{P}_2$ are unlikely to be greatly affected and their levels will reflect markedly reduced steady-state mass levels of $\text{PtdIns}(4,5)\text{P}_2$ and $\text{PtdIns}(4)\text{P}$. The ability of PDBu to attenuate both carbachol-stimulated accumulation of $[\text{H}]\text{InsP}_x$ and depletion of $[\text{H}]\text{PtdIns}(4)\text{P}$ and $[\text{H}]\text{PtdIns}(4,5)\text{P}_2$ suggests reduced PIC activity. Although enhancement of inositol phospholipid kinase activities by PDBu may also result in smaller changes in $\text{PtdIns}(4)\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$, the kinases involved would have to be co-ordinately regulated and it seems more likely that PDBu directly or indirectly modifies PIC activity.

PDBu inhibition of phosphoinositide signalling occurs, at least in part, at the post-receptor level

Over a period of 60 min, PDBu had no effect upon the binding of $[\text{H}]\text{NMS}$ to muscarinic M_3 receptors expressed in CHO cells under conditions equivalent to those used in our other experimental protocols (results not shown). Thus, PDBu does not reduce the number of cell-surface muscarinic receptors during short periods of exposure. It is unclear, however, if the effect of PDBu on phosphoinositide signalling occurs through modification of the receptor and/or other post-receptor effectors. This was investigated in cells permeabilized by electroporation in which $\text{Ins}(1,4,5)\text{P}_3$ accumulation could be induced by receptor-dependent (activation of muscarinic receptors) and receptor-independent (GTP[S] activation of G-proteins) methods.

Both carbachol and GTP[S] stimulated a time-dependent

Figure 4 PDBu attenuated the carbachol-mediated accumulation of $[^3\text{H}]\text{InsP}_3$ in $[^3\text{H}]\text{inositol}$ prelabelled cells with either a Li^+ -block of inositol monophosphatase activity (A) or incubated with 10 mM inositol to block recycling of $[^3\text{H}]\text{inositol}$ (B); PDBu also limited the carbachol-mediated depletion of $[^3\text{H}]\text{GroPIns}(4,5)\text{P}_2$ (C) and $[^3\text{H}]\text{GroPIns}(4)\text{P}$ (D) in cells with an inositol block

Time course of accumulation of $[^3\text{H}]\text{InsP}_3$ under Li^+ block (A) following addition of 1 mM carbachol to cells with (□) or without (■) 5 min pretreatment with 1 μM PDBu. PDBu caused a significant inhibition of accumulation of $[^3\text{H}]\text{InsP}_3$ ($P = 0.0009$, $n = 3$). Time courses of accumulation of $[^3\text{H}]\text{InsP}_3$ (B) and depletion of $[^3\text{H}]\text{GroPIns}(4,5)\text{P}_2$ (C) and $[^3\text{H}]\text{GroPIns}(4)\text{P}$ (D) under inositol block following addition of 1 mM carbachol (■, □) or buffer alone (▲, △) with (□, △) or without (■, ▲) 5 min pretreatment with 1 μM PDBu. PDBu was associated with a significant inhibition of both $[^3\text{H}]\text{InsP}_3$ accumulation ($P = 0.012$, $n = 4$) and depletion of $[^3\text{H}]\text{GroPIns}(4,5)\text{P}_2$ ($P = 1.38 \times 10^{-6}$, $n = 4$) and $[^3\text{H}]\text{GroPIns}(4)\text{P}$ ($P = 2.21 \times 10^{-6}$, $n = 4$).

accumulation of $\text{Ins}(1,4,5)\text{P}_3$ in permeabilized cells (Figure 5A). In contrast to the response in intact cells, carbachol mediated a slower increase in $\text{Ins}(1,4,5)\text{P}_3$ which peaked at 120 s and then declined to a sustained component. The increase in $\text{Ins}(1,4,5)\text{P}_3$ following addition of GTP[S] displayed different kinetics such that there was a lag following addition of GTP[S] and a peak at 180 s. (This level was then sustained for a further 120 s.) The slower rate of $\text{Ins}(1,4,5)\text{P}_3$ accumulation in response to GTP[S] may be due to the dependence on basal GTP/GDP exchange [28], whereas this will be facilitated by muscarinic receptor activation leading to a more rapid activation of PIC.

Treatment of permeabilized cells with PDBu had no effect on basal $\text{Ins}(1,4,5)\text{P}_3$ (24.5 ± 5.0 control, 30.1 ± 3.9 PDBu-treated, pmol/mg of protein, $n = 7$). However, PDBu significantly ($P <$

0.001) reduced the maximal $\text{Ins}(1,4,5)\text{P}_3$ response to carbachol (93.3 ± 10.2 versus 146.5 ± 11.3 , $n = 7$) demonstrating that, after permeabilization, PDBu-sensitive elements of $\text{Ins}(1,4,5)\text{P}_3$ accumulation were retained. This further supports a primary effect of PDBu on $\text{Ins}(1,4,5)\text{P}_3$ generation rather than the $[\text{Ca}^{2+}]_i$ response as the $[\text{Ca}^{2+}]_i$ is effectively clamped to that of the buffer (300 nM). The maximal $\text{Ins}(1,4,5)\text{P}_3$ response to GTP[S] was also significantly attenuated by PDBu (Figure 5B). Thus at least a component of PDBu sensitivity is at the post-receptor level in these cells. This is in contrast to previous studies indicating a direct effect of phorbol esters on muscarinic M_3 receptors in HT-29 cells [14] and PIC-linked muscarinic receptors of astrocytes [13]. However, data from the current study agree with previous reports of the inhibition of phosphoinositide responses by

Figure 5 Carbachol- or GTP[S]-mediated accumulation of Ins(1,4,5) P_3 in electroporabilized cells

(A) Time course of accumulation of Ins(1,4,5) P_3 in electroporabilized cells challenged with either 1 mM carbachol (■) or 100 μM GTP[S] (□). Data are means $\pm$ S.E.M., $n = 3$. (B) Effect of 1 μM PDBu or 10 μM Ro-318220 on Ins(1,4,5) P_3 accumulation in electroporabilized cells in response to 100 μM GTP[S]. Data are means $\pm$ S.E.M., $n = 4-7$, * $P < 0.05$ and ** $P < 0.01$ versus cells challenged with GTP[S] in the presence of PDBu (by Duncan's multiple-range test following one-way ANOVA with $P = 0.0012$).

phorbol esters at the post-receptor level [8,11]. As $G_{\alpha_{q/11}}$ is not phosphorylated in response to either PDBu or agonist [8], inhibition is unlikely to be at this level. However, PIC- β , which probably mediates muscarinic receptor-mediated hydrolysis of PtdIns(4,5) P_2 , is a substrate for PKC [29]. Although this phosphorylation does not affect catalytic activity, it may alter interaction with $G_{\alpha_{q/11}}$, thereby limiting activation [29].

As the kinetics of Ins(1,4,5) P_3 accumulation in permeabilized cells differed between carbachol and GTP[S], the proportion of PDBu sensitivity attributable to events at the post-receptor level could not be estimated. Phosphorylation of the receptor itself may be associated with desensitization [4] and it is possible that PDBu sensitivity could also occur at the receptor following phosphorylation by PKC. Indeed human muscarinic M_3 receptors expressed in CHO cells are a substrate for PKC [30] although in those cells PKC may not mediate the major part of agonist-sensitive phosphorylation [31].

Table 2 Ro-318220 protected against the effects of PDBu but alone did not influence Ins(1,4,5) P_3 responses to carbachol

Ins(1,4,5) P_3 responses to 1 mM carbachol in control (untreated) cells and cells treated prior to agonist addition with 1 μM PDBu for 5 min, 10 μM Ro-318220 for 10 min or 10 μM Ro-318220 for 10 min plus 1 μM PDBu for 5 min. Data are Ins(1,4,5) P_3 levels in pmol/mg of protein, mean $\pm$ S. E. M., $n = 3$, $P < 0.01$ for * versus other groups (by Duncan's multiple range test following one-way ANOVA with $P = 0.039$).

	Control	PDBu	Ro-318220	Ro-318220 + PDBu
Basal	18.8 $\pm$ 3.2	18.3 $\pm$ 3.3	15.9 $\pm$ 3.0	12.8 $\pm$ 2.4
Peak (10 s)	181.9 $\pm$ 29.3	95.4 $\pm$ 7.1*	151.9 $\pm$ 19.9	143.1 $\pm$ 20.1
Plateau (300 s)	67.1 $\pm$ 2.0	43.0 $\pm$ 2.0	57.3 $\pm$ 8.7	60.5 $\pm$ 5.4

PDBu but not carbachol activation of PKC attenuates phosphoinositide and $[\text{Ca}^{2+}]_i$ signalling

Treatment with the PKC inhibitor, Ro-318220 (10 μM), for 10 min had no effect on carbachol-induced peak or sustained Ins(1,4,5) P_3 elevations (Table 2) or peak $[\text{Ca}^{2+}]_i$ elevations (results not shown). However, treatment with Ro-318220 prevented the ability of PDBu to reduce carbachol-mediated peak Ins(1,4,5) P_3 (Table 2) and $[\text{Ca}^{2+}]_i$ (results not shown) responses. The carbachol-induced sustained elevations of Ins(1,4,5) P_3 were not significantly affected by these treatments although the pattern of change reflected those seen in peak Ins(1,4,5) P_3 levels. These treatments did not affect sustained $[\text{Ca}^{2+}]_i$ responses to carbachol (results not shown). Combined with the inability of Ro-318220 to potentiate GTP[S]-stimulated Ins(1,4,5) P_3 accumulation in permeabilized cells (Figure 5B) these results indicate a lack of PKC feedback regulation of phosphoinositide signalling unless PKC is activated by PDBu.

Ins(1,4,5) P_3 accumulation remained biphasic in cells treated with Ro-318220 (results not shown). This observation and the lack of effect of PDBu on the shape of the biphasic profile of Ins(1,4,5) P_3 accumulation and $[\text{H}^3]\text{Ins}P_x$ accumulation (in Li^+ -blocked cells), indicates that PKC activation does not underlie the rapid desensitization of PIC activity that occurs within seconds of agonist exposure. This desensitization may, therefore, be related to phosphorylation of the muscarinic receptor by a receptor-specific kinase or other mechanisms such as the rapid, sustained and marked depletion of PtdIns(4,5) P_2 (Figure 4C) [4].

Inhibition by PKC inhibitors of $[\text{H}^3]\text{NMS}$ binding to human muscarinic M_3 receptors

Although the above data indicate a lack of agonist-mediated PKC feedback at maximal concentrations of carbachol, at submaximal agonist concentrations, when PIC is not maximally activated, such feedback may be relevant. Protocols designed to examine this would require the use of Ro-318220 at submaximal agonist concentrations. However, studies within our laboratory had indicated that Ro-318220 may be a muscarinic antagonist. Thus, the inhibition of $[\text{H}^3]\text{NMS}$ binding by Ro-318220 and a number of other PKC inhibitors having differing modes of action [32] was assessed.

All of the PKC inhibitors reduced $[\text{H}^3]\text{NMS}$ binding (Figure 6). Assuming competitive interaction and using the Cheng-Prusoff factor [33] Ro-318220 and chelerythrine chloride had estimated K_d values ($\log_{10}$, mean $\pm$ S.E.M., $n = 3$) at the muscarinic M_3 receptor of -4.83 ± 0.04 (15 μM) and -5.27 ± 0.12 (5 μM) respectively.

Ro-318220 did not affect Ins(1,4,5) P_3 or $[\text{Ca}^{2+}]_i$ levels (results not shown) indicating antagonist activity. Given an EC_{50} for

Figure 6 Effect of inhibitors of PKC on the binding of [3 H]NMS to recombinant human muscarinic M_3 receptors expressed in CHO cells

Specific binding of [3 H]NMS as a percentage of control in the presence of the indicated concentrations of Ro-318220 ($\square$), chelerythrine chloride ($\blacktriangle$) and calphostin-C ($\triangle$). Data are means $\pm$ S.E.M., $n = 3$, see text for estimates of K_d values for Ro-318220 and chelerythrine chloride.

carbachol-mediated peak $\text{Ins}(1,4,5)P_3$ responses in SH-SY5Y cells of 25 μM [16], the Cheng–Prusoff factor predicts an IC_{50} for Ro-318220 of 615 μM at 1 mM carbachol. At 10 μM Ro-318220 such inhibition will be of little consequence. However, at 25 μM carbachol, the IC_{50} for Ro-318220 would be 30 μM and thus 10 μM Ro-318220 may well compromise agonist-stimulated responses. Antagonism [3 H]NMS binding was not restricted to Ro-318220 and may not be specific to muscarinic M_3 receptors. This provides a cautionary note when inhibitors are used to assess the role of agonist-mediated PKC activation.

Effects of long-term PDBu treatment on phosphoinositide signalling

To overcome the antagonistic activity of Ro-318220 at the muscarinic receptor at submaximal agonist concentrations we sought to down-regulate PKC by long-term PDBu treatment. This has been reported to down-regulate at least some isoforms of PKC [12,14,34,35]. Thus, if agonist activation of PKC mediates negative feedback, then down-regulation may enhance PIC activity.

Exposure to PDBu for either 24 h or 5 min prior to carbachol did not affect basal levels of $\text{Ins}(1,4,5)P_3$ (12.8 ± 4.1 , $n = 9$) but significantly reduced peak responses. This occurred at maximal (1 mM) concentrations of carbachol (24 h, 139.2 ± 7.9 ; 5 min, 125.8 ± 17.7 ; untreated, 235.0 ± 17.7 , $P < 0.05$) and at the EC_{50} (25 μM) (24 h, 54.2 ± 8.0 ; 5 min, 69.5 ± 14.6 ; untreated,

107.8 ± 14.3 , $n = 3$, $P < 0.01$, all data expressed as pmol/mg of protein, $n = 3$).

The current study was in marked contrast to those reporting potentiation of agonist-mediated phosphoinositide responses following long-term phorbol ester treatment [34,35]. Such an effect is not unprecedented [36] and may well be receptor- and/or cell-dependent [37]. This may result from the differential down-regulation of PKC isoforms by phorbol esters and it is possible that in the current study the isoform responsible for inhibition was not sufficiently down-regulated. However, loss of PKC is unlikely to be the only consequence of long-term phorbol ester treatment. Indeed in SH-SY5Y cells, such a protocol resulted in a loss of [3 H]NMS binding [38]. Whether other components of signalling pathways are affected is unclear but interpretation of altered phosphoinositide responses following this protocol is very difficult.

Conclusions

The current study indicates that the activation of PKC by PDBu inhibits muscarinic receptor-mediated $\text{Ins}(1,4,5)P_3$ accumulation and $[\text{Ca}^{2+}]_i$ elevation by reducing the hydrolysis of $\text{PtdIns}(4,5)P_2$ at least partially at the post-receptor level. The rapid, transient phases of the biphasic responses are preferentially affected. Although we cannot eliminate an effect at sub-maximal agonist concentrations, the data provide no evidence of an effect of agonist-mediated PKC activation on phosphoinositide and $[\text{Ca}^{2+}]_i$ signalling. This is in contrast to findings with a number of other receptor types [8,39–42] and also an indication that in some instances PKC, following direct activation of G-proteins, can inhibit phosphoinositide responses [8].

Divergence between phorbol ester-mediated and agonist-mediated PKC activation may, however, be a common phenomenon. Thus, while phorbol esters desensitized PIC-linked responses to bombesin in Swiss 3T3 fibroblasts [43] and histamine in immortalized hypothalamic GT1-7 neuronal cells [44], PKC was not involved in agonist-mediated desensitization. The ability of PKC, activated by means other than phorbol ester treatment, to influence PIC may therefore be dependent upon both cell and receptor type through, for example, differences in the level or isoforms of PKC and PIC expressed and/or the susceptibility of receptors to agonist- and PKC-dependent phosphorylation.

Although the association between translocation and activation should be made with caution, the current study suggested that PDBu provoked a greater activation of PKC than carbachol. Whether this difference is sufficient to account for the ability of PDBu, but not carbachol, to activate a PKC feedback loop is unclear. Aside from a more selective activation of isoenzymes other than $\text{PKC}\alpha$ by PDBu compared with carbachol, differences in the time course of PKC activation may also be important. Thus, when PDBu was used, PKC was maximally activated (assessed by translocation of $\text{PKC}\alpha$) at the point of agonist addition. However, maximal activation of PKC by carbachol may be delayed and peak of $\text{Ins}(1,4,5)P_3$ accumulation, which shows greatest sensitivity to PDBu, occurs prior to maximal activation of PKC. An existing agonist-mediated PKC activation may, however, limit activation of PIC by other receptor types. The combination of a lower sensitivity to PKC and a lower level of PKC activation by carbachol compared with PDBu could account for the lack of agonist-mediated PKC feedback during sustained responses. In the context of apparent Ca^{2+} and PKC-sensitive components of agonist-mediated $\text{Ins}(1,4,5)P_3$ accumulation (see above) it is also interesting to speculate that agonist-mediated PKC feedback could be of more importance when Ca^{2+} facilitation of PIC is reduced or absent, for example during an

ongoing depletion of the rapidly releasable intracellular Ca²⁺ pools [23].

This work was supported by a programme grant from the Wellcome Trust (ref. 16895/91).

REFERENCES

- Berridge, M. J. (1993) *Nature (London)* **361**, 315–325
- Mullaney, I., Dodd, M. W., Buckley, N. and Milligan, G. (1993) *Biochem. J.* **289**, 125–131
- Wojcikiewicz, R. J. H., Furuichi, T., Nakade, S., Mikoshiba, K. and Nahorski, S. R. (1994) *J. Biol. Chem.* **269**, 7963–7969
- Wojcikiewicz, R. J. H., Tobin, A. B. and Nahorski, S. R. (1993) *Trends Pharmacol. Sci.* **14**, 279–285
- Wojcikiewicz, R. J. H., Tobin, A. B. and Nahorski, S. R. (1994) *J. Neurochem.* **63**, 177–185
- Fisher, S. K., Slowiejko, D. W. and McEwen, E. L. (1994) *Neurochem. Res.* **19**, 549–554
- Menniti, F. S., Takemura, H., Oliver, K. G. and Putney, Jr., J. W. (1991) *Mol. Pharmacol.* **40**, 727–733
- Barr, A. J. and Watson, S. P. (1994) *Mol. Pharmacol.* **46**, 266–273
- Labarca, R., Janowsky, A., Patel, J. and Paul, S. M. (1984) *Biochem. Biophys. Res. Commun.* **123**, 703–709
- Vincentini, L. M., Virgilio, F. D., Ambrosini, A., Pozzan, T. and Meldolesi, J. (1985) *Biochem. Biophys. Res. Commun.* **127**, 310–317
- Orellana, S., Soliski, P. and Brown, J. H. (1987) *J. Biol. Chem.* **262**, 1638–1643
- Lai, W. S. and El-Fakahany, E. E. (1988) *J. Pharmacol. Exp. Ther.* **244**, 41–50
- Pearce, B., Morrow, C. and Murphy, S. (1988) *J. Neurochem.* **50**, 936–944
- Kopp, R., Mayer, P. and Pfeiffer, A. (1990) *Biochem. J.* **269**, 73–78
- Murphy, N. P., McCormack, J. G., Ball, S. G. and Vaughan, P. F. T. (1992) *Biochem. J.* **282**, 645–650
- Willars, G. B. and Nahorski, S. R. (1995) *Br. J. Pharmacol.* **114**, 1133–1142
- Lambert, D. G., Ghataore, A. S. and Nahorski, S. R. (1989) *Eur. J. Pharmacol.* **165**, 71–77
- Gryniewicz, G., Poenie, M. and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Lowry, O. H., Rosebrough, L. W., Farr, L. W. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Jenkinson, S., Nahorski, S. R. and Challiss, R. A. J. (1994) *Mol. Pharmacol.* **46**, 1138–1148
- Burford, N. T., Tobin, A. B. and Nahorski, S. R. (1995) *J. Pharmacol. Exp. Ther.* **274**, 134–142
- Leli, U., Shea, T. B., Cataldo, A., Hauser, G., Grynspan, F., Beerman, M. L., Liepkalns, V. A., Nixon, R. A. and Parker, P. J. (1993) *J. Neurochem.* **60**, 289–298
- Willars, G. B. and Nahorski, S. R. (1995) *Mol. Pharmacol.* **47**, 510–517
- Lambert, D. G. and Nahorski, S. R. (1990) *Biochem. J.* **265**, 555–562
- Eberhard, D. A. and Holz, R. W. (1988) *Trends Neurosci.* **11**, 517–520
- Conway, B. R., Withiam-Leitch, M. and Rubin, R. P. (1993) *Mol. Pharmacol.* **43**, 286–292
- Snoek, G. T., Westerman, J., Wouters, F. S. and Wirtz, K. W. A. (1993) *Biochem. J.* **291**, 649–656
- Kaziro, Y., Itoh, H., Kozasa, T., Nakafuku, M. and Satoh, T. (1991) *Annu. Rev. Biochem.* **60**, 349–400
- Ryu, S. H., Kim, U.-H., Wahl, M. I., Brown, A. B., Carpenter, G., Huang, K.-P. and Rhee, S. G. (1990) *J. Biol. Chem.* **265**, 17941–17945
- Tobin, A. B. and Nahorski, S. R. (1993) *J. Biol. Chem.* **268**, 9817–9823
- Tobin, A. B., Keys, B. and Nahorski, S. R. (1993) *FEBS Lett.* **335**, 353–357
- Azzi, A., Boscoboinik, D. and Hensey, C. (1992) *Eur. J. Biochem.* **208**, 547–557
- Hulme, E. C. and Birdsall, N. J. M. (1992) in *Receptor-ligand Interactions — A Practical Approach* (Hulme, E. C., ed.), pp. 63–176, Oxford University Press, Oxford
- Hepler, J. R., Earp, H. S. and Harden, T. K. (1988) *J. Biol. Chem.* **263**, 7610–7619
- Pfeilschifter, J., Ochsner, M., Whitebread, S. and De Gasparo, M. (1989) *Biochem. J.* **262**, 285–291
- Jones, J. A., Owen, P. J. and Boarder, M. R. (1990) *Br. J. Pharmacol.* **101**, 521–526
- Lin, W.-W., Lee, C. Y. and Chuang, D.-M. (1990) *Biochem. Biophys. Res. Commun.* **168**, 512–519
- Cioffi, C. L. and Fisher, S. K. (1990) *J. Neurochem.* **54**, 1725–1734
- King, W. G. and Rittenhouse, S. E. (1989) *J. Biol. Chem.* **264**, 6070–6074
- Boarder, M. R. and Challiss, R. A. J. (1992) *Br. J. Pharmacol.* **107**, 1140–1145
- Challiss, R. A. J., Wilkes, L. C., Patel, V., Purkiss, J. R. and Boarder, M. R. (1993) *FEBS Lett.* **327**, 157–160
- Alblas, J., van Etten, I., Khanum, A. and Moolenaar, W. H. (1995) *J. Biol. Chem.* **270**, 8944–8951
- Walsh, J. H., Bouzyk, M. and Rozengurt, E. (1993) *J. Cell. Physiol.* **156**, 333–340
- Zamani, M. R., Dupere, R. B. and Bristow, D. R. (1995) *J. Neurochem.* **65**, 160–169