Biochemical Pharmacology, Vol. 45, No. 3, pp. 611–617, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

THE EFFECTS OF β-PHORBOL-12,13 DIBUTYRATE ON AGONIST-INDUCED INCREASES IN $[Ca^{2+}]_i$ IN N1E-115 CELLS

DIFFERENTIAL MODULATION OF RESPONSES TO ANGIOTENSIN II AND BRADYKININ

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(*Received* 21 *September* 1992; *accepted* 29 *October* 1992)

Abstract—Addition of angiotensin II (A2; 500 nM) to populations of fura-2-loaded N1E-115 cells resulted in a transient increase in intracellular calcium which was abolished by pre-treatment with the phorbol ester, β-phorbol-12,13 dibutyrate (PDBu) (1.5 μM). The inhibitory effects were reversed by the protein kinase C inhibitor, staurosporin (150 nM), and down-regulation of protein kinase C was observed over 48 hr. Responses to maximally effective concentrations of histamine (300 μM), ATP (100 μM), UTP (100 μM) and carbachol (100 μM) were similarly inhibited by phorbol pre-treatment but the response to bradykinin (BK) (100 nM) was unaffected. When the concentrations of BK and A2 were adjusted to produce the same-sized calcium signals, PDBu pre-treatment abolished the response to A2 but only partially inhibited the response to BK. From the data presented here we can conclude that the calcium response to BK in N1E-115 cells is less susceptible to the inhibitory effects of protein kinase C activation than the response produced by A2.

The binding of an agonist with its receptor in a number of cell lines activates phosphoinositidase C which in turn catalyses the breakdown of phosphatidylinositol-4,5-bisphosphate releasing inositol-1,4,5-trisphosphate ($InsP_3$‡) and diacylglycerol [1–4]. In turn, $InsP_3$ mobilizes intracellular calcium ($[Ca^{2+}]_i$) from storage sites, which can be monitored by the use of fluorescent dyes [5–7].

Diacylglycerol itself plays a secondary messenger role via activation of protein kinase C [8]. Furthermore, activation of protein kinase C by phorbol esters [9–11] has been shown to attenuate agonist-stimulated elevations in $[Ca^{2+}]_i$ [12–14]. Several possible modes of action of this feedback control mechanism have been proposed including receptor or G protein phosphorylation [15, 16], activation of an $InsP_3$ phosphatase [17], blockade of the intracellular site of release [18] and activation of a Ca^{2+} transport ATPase and Na^+/Ca^{2+} exchange [19].

The murine neuroblastoma clone N1E-115 is known to possess a number of receptors linked to mobilization of $[Ca^{2+}]_i$ [20–23]. However, it is not known whether all these calcium responses are similarly affected by pre-treatment with phorbol esters. In the present study, comparisons were made between a range of agonists and we report that calcium mobilisation is *differentially* inhibited by protein kinase C activation.

MATERIALS AND METHODS

N1E-115 cells, passages 17–26, were cultured in 75 cm^2 flasks in Dulbecco's modified Eagle's medium (with glutamine) containing 5% foetal calf serum (FCS) without antibiotics. The monolayers from two near-confluent flasks were detached using Pucks D1 solution (glucose, 5.5 mM; KCl, 5.4 mM; sucrose, 58.4 mM; Na_2HPO_4, 0.17 mM; NaCl, 138 mM; and KH_2PO_4, 0.22 mM) and resuspended in a simple saline HEPES buffer ($CaCl_2$, 2 mM; NaCl, 145 mM; glucose, 10 mM; KCl, 5 mM; $MgSO_4$, 1 mM; and HEPES, 10 mM; pH 7.45). This was followed by incubation with fura-2 acetoxy methyl ester (5 μM) at 37° (in the presence of 5% FCS) for 20 min and for a further 5 min following a 3-fold dilution (to ensure maximum hydrolysis of ester to the acid form). At the end of this loading period, excess dye was removed by centrifugation and the cells were resuspended in fresh buffer (no serum) and left at room temperature until use. Each run was preceded by a rapid spin in a microcentrifuge followed by resuspension in fresh buffer (at an approximate cell concentration of 5×10^5/mL).

All experiments were carried out using a Perkin–Elmer LS 50 Spectrometer, with excitation ratioing between 340 and 380 nm, and recording at 500 nm. The time course for each run was 200 sec with drugs added in 10 μL (bradykinin 16 μL) aliquots.

Calibration. At the end of a run, ionomycin (20 μM) was added followed by EGTA (6.25 mM, pH greater than 8.5) in order to calculate R_{max} and

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‡ Abbreviations: $InsP_3$, inositol 1,4,5-trisphosphate; $[Ca^{2+}]_i$, intracellular calcium; FCS, foetal calf serum; A2, angiotensin II; BK, bradykinin; PDBu, β-phorbol-12,13 dibutyrate; SS, staurosporin.

R_{min}. Autofluorescence was determined using a separate cuvette following the addition of manganese (5 mM) after the ionophore ionomycin (20 μM). Using these values and those obtained with fura-2 free acid, $[Ca^{2+}]_i$ was calculated according to the method of Grynciewicz *et al.* [24].

Phorbol experiments. For short period pre-incubation studies, β-phorbol-12,13 dibutyrate PDBu or 4-α-phorbol (1.5 μM) was added for the duration of the 25 min dye-loading period. For down-regulation experiments, PDBu was added to the incubation media 48 hr prior to experiments being carried out. When appropriate, staurosporin (SS; 150 nM) was added 1 hr before cells were detached from the flasks and then maintained throughout the whole experiment.

Measurement of $InsP_3$. This was carried out using minor modifications of the radioreceptor method as described previously [25]. Briefly, N1E-115 cells (approx. 5×10^5 cells/mL) were preincubated in simple saline buffer for 10 min at 37°. Initially, aliquots of the cell suspension were removed at intervals in order to measure basal $InsP_3$. The agonist was then added and further aliquots taken at specific time points (initially every 10 sec). Ice-cold perchloric acid (7.5%) was used to stop the reaction (at each time point), the samples were neutralized with a calibrated amount of $KHCO_3$ (1.2 M) and the protein was separated by centrifugation. $InsP_3$ was quantified in the supernatant layer by a radioreceptor assay using a bovine adrenal–cortical binding protein at 4°. Authentic $InsP_3$ (10^{-10}–10^{-14} mol) in neutralized perchloric acid buffer was used to construct a standard curve for displacement of bound [^{3}H]$InsP_3$. The bound [^{3}H]$InsP_3$ was separated by rapid filtration and quantified by liquid scintillation spectrometry. The protein pellet was digested in NaOH (0.5 M) and estimated by the method of Bradford [26].

Materials. N1E-115 cells were supplied by Porton Down. Cell culture flasks were obtained from Costar with Dulbecco's modified Eagle's medium and FCS from NBL Ltd (Cramlington, U.K.). PDBu, 4-α-phorbol, bradykinin (BK), angiotensin II (A2), histamine and carbachol were supplied by the Sigma Chemical Co. (Poole, U.K.), with fura-2 AM and ionomycin from Calbiochem (Novobiochem, Nottingham, U.K.). $InsP_3$ was obtained from Amersham (U.K.) and [^{3}H]$InsP_3$ from NEN Dupont (Stevenage, U.K.). ATP and UTP were supplied by Boehringer Mannheim (Lewes, U.K.).

Statistics and data analysis. EC_{50} and IC_{50} (concentrations of drug producing 50% of maximum stimulation and inhibition) values were obtained by computer-assisted curve fitting by use of the computer program Graph-Pad (ISI). Calcium data was captured using the ICBC program supplied by Perkin–Elmer and imported to the graphics program Sigma-Plot (Jandel).

Significance testing was carried out using an unpaired Student's *t*-test or one-way analysis of variance with a post-hoc Newman–Keuls test.

RESULTS

The effect of PDBu on the calcium response to A2

A2 (500 nM; the maximally effective concentration) produced a rapid and transient increase in $[Ca^{2+}]_i$ of 203 ± 29 nM from a resting value of 106 ± 9 nM (N = 3; Fig. 1A). The effects were found to be concentration-dependent (Fig. 1B). Preincubation with PDBu for 20 min (1.5 μM) resulted in a significant reduction in this response to 6 ± 9% (N = 5; $P < 0.01$) of the control value (Fig. 2A and B). The inhibitory effect was concentration dependent with a log IC_{50} value of −7.0 ± 0.06 (Fig. 2C).

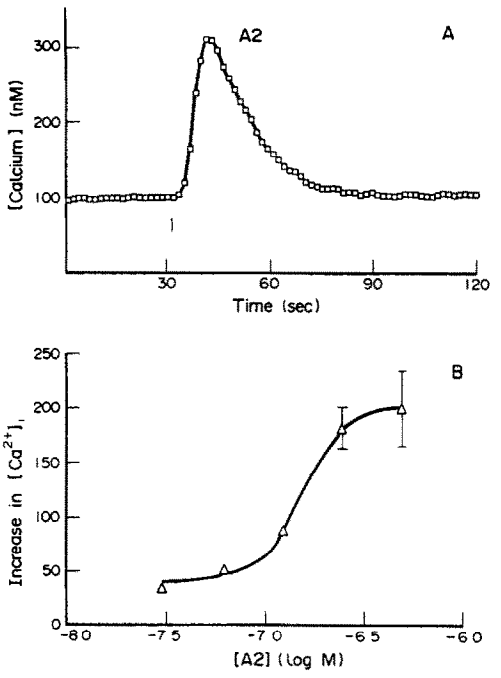

Fig. 1. (A) The effect of A2 (500 nM) on $[Ca^{2+}]_i$ in populations of fura-2-loaded N1E-115 cells. Fluorescence values were measured following excitation at 340 and 380 nm and the ratio used to determine $[Ca^{2+}]_i$. The graph is typical of two others. (B) The concentration–response relationship for A2. The graph shows increases in $[Ca^{2+}]_i$ (nM) as a function of [A2] (log M). Data are means of three separate determinations; vertical error bars represent SEM.

The effect of 4-α-phorbol on the A2 response

Pre-treatment with the inactive phorbol, 4-α-phorbol (1.5 μM), was without effect on the A2 response (102 ± 3% of controls; N = 3).

The effect of PDBu on the responses to other agonists

Pre-incubation with PDBu (1.5 μM) resulted in significant (all $P < 0.05$) reductions in calcium responses to maximally effective concentrations of all agents investigated, *with the exception of BK* (Table 1). No significant effect was seen on the response to 100 nM BK (95 ± 7% of controls, N = 4), but the increase in $[Ca^{2+}]_i$ elicited by 50 nM BK was significantly reduced to 79 ± 10% of controls ($P < 0.05$; N = 4).

When the concentration of BK was reduced (to 4.4 nM) so that the calcium signal produced matched

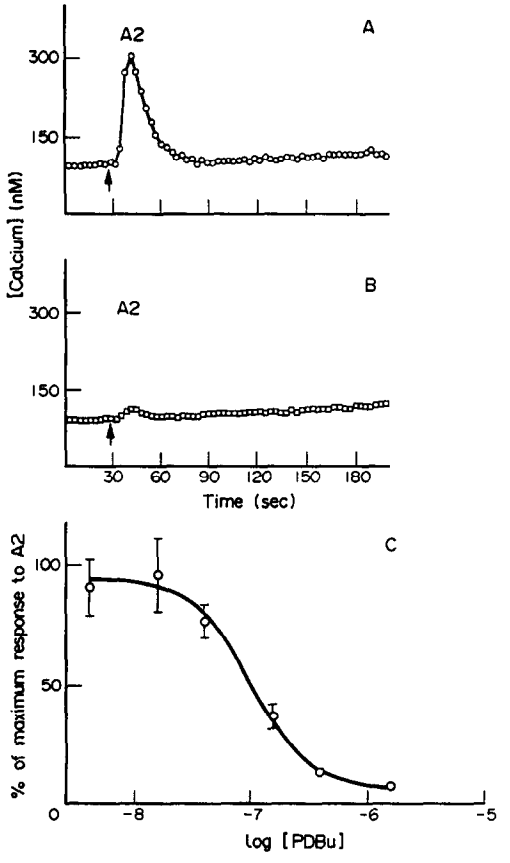

Fig. 2. The effect of PDBu (1.5 μM) pre-treatment on the A2-induced calcium response. PDBu was present during the whole of the experiment. The upper two panels show the response to A2 (500 nM) with (B) and without (A) phorbol pre-treatment. Each trace is typical of two others. (C) The concentration–response relationship for PDBu. The concentration of A2 used was 500 nM. Data are means of three separate determinations; vertical error bars represent SEM.

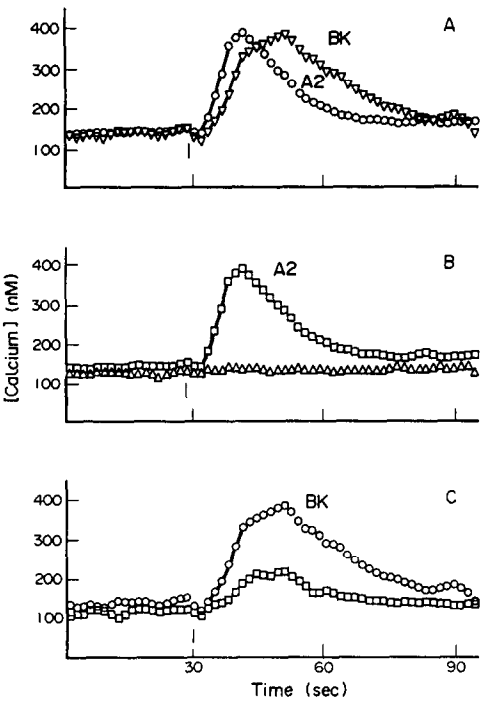

Fig. 3. The differential phorbol sensitivities of calcium responses to A2 and BK. (B) The effect of PDBu (1.5 μM) on the calcium response to a maximally effective concentration of A2 (500 nM; squares absence of PDBu, triangles presence of PDBu). (C) The effect of PDBu on the calcium response to a sub-maximally effective concentration of BK (4.4 nM; circles absence of PDBu, squares presence of PDBu). The concentrations of the two agonists were chosen to produce identically sized calcium responses in the absence of phorbol pre-treatment (A). Each of the traces shown is typical of two others.

Table 1. The effects of PDBu (1.5 μM) pre-treatment on maximal calcium responses to various agonists

Agonist	Concn	% control response	N
BK	100 nM	95 ± 7	4
A2	500 nM	6 ± 9	5
Carbachol	100 nM	22 ± 16	4
Histamine	300 μM	10 ± 10	6
ATP	100 μM	36 ± 5	4
UTP	100 μM	42 ± 4	3

The data are shown as percentages of the control responses determined in the absence of phorbol and are the means of three to six separate determinations; errors indicated are SEM.

that produced by a maximal concentration of A2 (Fig. 3A), there was still a significant difference in the sensitivity to phorbol pre-treatment between the two agonists (Fig. 3B and C). Thus, the response to A2 was abolished under conditions where a significant BK response was still observed ($N = 3$; $P < 0.05$).

Pre-treatment with staurosporin

Pre-incubation with the protein kinase C inhibitor, SS (150 nM; 60 min), significantly attenuated the effects of PDBu on the A2 response ($P < 0.01$; Fig. 4). However, pre-treatment with SS alone produced a small reduction in the calcium response to A2 (to 71 ± 1% of controls; $P < 0.001$, $N = 3$). SS apparently failed to reverse completely the phorbol-induced inhibition although, when the inhibitory effect of SS itself is taken into account, it is clear that the inhibition was totally prevented by the protein kinase C inhibitor. The reason for the inhibitory effect of SS alone is not clear but in common with other protein kinase C inhibitors, such as H7 and H8, SS is not specific and is known to affect other intracellular kinases [27, 28].

Down-regulation of the response to PDBu

Pre-treatment for 48 hr of the cells with PDBu

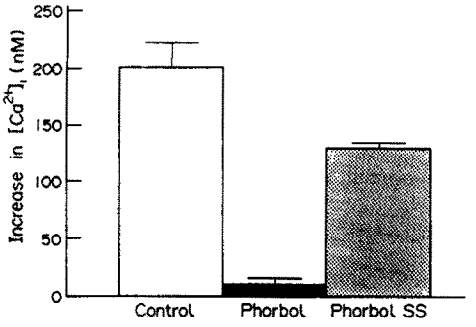

Fig. 4. Reversal of the phorbol inhibition with SS. The cells were pre-treated with SS (150 nM) for 1 hr prior to disruption of their monolayers (see text). The bar graph shows the effects of PDBu (1.5 μM) alone and PDBu with SS on the control response to A2 (500 nM). Pre-treatment with SS reversed the phorbol inhibition, returning A2 responses to 65% of control values. Data are means of three separate determinations; error bars represent SEM. For significance testing, data were analysed by one-way analysis, with comparisons between all pairs of data using the Newman–Keuls test. SS reversed the inhibitory effects of PDBu ($P < 0.01$).

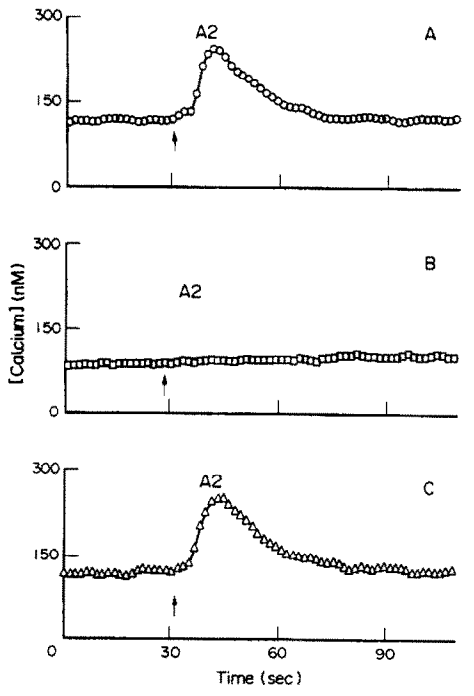

Fig. 5. Down-regulation of the phorbol inhibition. Cells were pre-treated with PDBu (1.5 μM) for 25 min or 48 hr (see text). (A) The control response in the absence of any treatment with PDBu; (B) the effect of 25 min pre-treatment with PDBu (as Fig. 2) and (C) 48 hr pre-treatment with PDBu followed by PDBu for an additional 25 min period. The inhibitory response was down-regulated following the 48 hr pre-treatment resulting in a lack of secondary phorbol effect. Each trace shown is typical of two others.

(1.5 μM) significantly attenuated the acute effects of PDBu on the A2-mediated $[Ca^{2+}]_i$ rise (Fig. 5).

The effect of PDBu on ATP-mediated calcium entry

PDBu (1.5 μM) caused a significant reduction in the response to ATP (36 ± 5% of control; 100 μM; $P < 0.01$, $N = 4$). However, the calcium response to 1 mM ATP (previously shown to cause a secondary entry of calcium [29]), added approximately 1 min later, was unaffected (95 ± 13% of control response).

The effect of PDBu on $InsP_3$ production

$InsP_3$ levels were successfully measured following stimulation with ATP and BK (the responses to A2 were not consistent). Pre-incubation with PDBu (1.5 μM) resulted in a significant ($P < 0.05$; results from 3 different days, each of three independent determinations) reduction in ATP-stimulated $InsP_3$ production (from 29 ± 5 to 14 ± 4 pmol/mg above basal levels; 52%). Similarly, phorbol pre-treatment resulted in a significant ($P < 0.05$; results from 2 different days, each of three independent determinations) but *smaller* reduction in BK-stimulated (100 nM; Fig. 6) $InsP_3$ production (from 67 ± 5 to 52 ± 4 pmol/mg above basal levels; 22%).

DISCUSSION

Stimulation of a number of receptor systems mobilizes calcium in N1E-115 cells [21, 23]. Evidence from studies using other cell lines suggests that mobilization of $[Ca^{2+}]_i$ can be inhibited by phorbol esters [15, 16]. In addition, 12-*O*-tetradecanoylphorbol-13-acetate has been shown to inhibit receptor-mediated cyclic GMP accumulation and inositol phospholipid hydrolysis [30, 31] in N1E-115 cells.

In the present study, addition of A2 to populations of fura-2-loaded N1E-115 cells resulted in a transient increase in $[Ca^{2+}]_i$ (Fig. 1) which was abolished following pre-treatment with PDBu (Fig. 2). The inhibition was significantly reversed by inclusion of the protein kinase C inhibitor, SS (Fig. 4), and was not observed in the presence of the inactive phorbol ester (4-α-phorbol). In addition, the effects of PDBu were down-regulated following prolonged exposure to the phorbol ester (48 hr; Fig. 5). It would therefore appear that PDBu exerts its effects via activation of protein kinase C. Increasing the concentration of A2 up to 0.1 mM failed to reverse the inhibitory effects of PDBu (data not shown) thereby indicating a non-competitive effect of the phorbol ester on receptor–effector coupling rather than a competitive blockade of the A2 recognition site.

A number of other agonists were assessed for sensitivity to PDBu pre-treatment. Calcium responses following stimulation with maximally effective concentrations of histamine, carbachol, ATP and UTP were significantly reduced, but the response to a maximally effective concentration of BK remained unchanged (Table 1). A similar differential sensitivity to phorbol treatment was seen between vasopressin and phenylephrine using 12-*O*-tetradecanoylphorbol-13-acetate in isolated hepa-

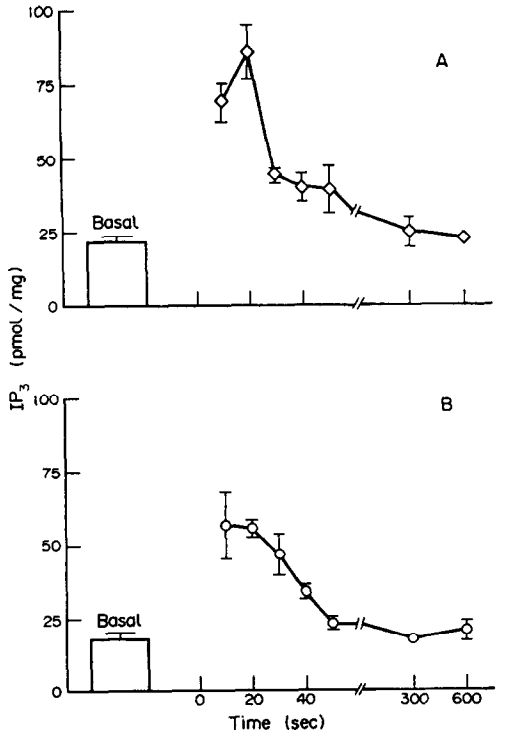

Fig. 6. The effects of PDBu (1.5 μM) on the $InsP_3$ response to BK (100 nM). The graphs show $InsP_3$ generation (pmol/mg) as a function of time (sec) in the presence (B) and absence (A) of PDBu. Data are means of three separate determinations; vertical error bars represent SEM.

tocytes [15]. However, the maximum calcium response to BK is much larger than that to A2; the reason for this is not certain but may involve a greater receptor density or more effective coupling of the BK receptor to calcium mobilization. Indeed, some sensitivity to PDBu was seen at sub-maximal concentrations of BK, with the response to 50 nM reduced by 16 ± 11% and 4.4 nM by 52 ± 9% (N = 3).

When the concentration of BK was reduced to produce a response which matched that caused by A2 a considerable difference in PDBu sensitivity was still apparent. Thus, the differential sensitivity for PDBu between the two agonists cannot be simply related directly to the size of the calcium response. It is also possible that BK and A2 are coupled to different pools of intracellular calcium which are affected to different extents by PDBu. However, additivity experiments with the two agonists have previously indicated that a common pool of calcium is employed (prior stimulation with BK reduced subsequent calcium mobilization due to A2; data not shown).

We have shown previously that the elevation of $[Ca^{2+}]_i$ in the N1E-115 cell line (except following stimulation with carbachol and millimolar ATP) does not appear to involve calcium influx and can be accounted for by calcium mobilization [29, 32], a possible target for the inhibitory effects of PDBu. However, it seems to be unlikely that the differential effect of PDBu is mediated at the level of $InsP_3$-induced calcium release, since it would be expected that matching calcium responses (presumably involving identical $InsP_3$ signals) be similarly affected by phorbol esters.

A number of other possible targets for protein kinase C phosphorylation have been proposed in different cell lines. Phosphorylation sites may exist at the level of the receptor, G protein or phosphoinositidase C. The observed differences in matching calcium responses to A2 and BK may be due to coupling of the receptors to different G proteins or the existence of a number of isozymes of phosphoinositidase C. Alternatively, if it is assumed that the same G protein and phosphoinositidase C isozyme are used by the two receptors, and the stoichiometry of coupling between them is the same (when the calcium responses are of equal magnitude), then similar sensitivity to PDBu would be expected, unless the receptors are the target for the differential effects of PDBu. However, there is evidence from radioligand binding studies that phorbol esters do not change the density of muscarinic receptors in N1E-115 cells [31] despite the fact that in the present study muscarinic receptor-mediated calcium mobilization was inhibited (Fig. 3). However, it is important to note that when measuring receptor binding in intact cell systems it is difficult to be certain that the ligand used is bound to surface proteins only and has not been taken up into the cell where it will bind to internalized receptors. Although the ligand used by Lai and El-Fakahany [31], [^{3}H]*N*-methylscopolamine, is reasonably hydrophilic further experiments would be needed to be certain of the conclusions drawn. One possible way of determining whether or not the ligand is taken up would be to compare the results obtained in a second study, using the highly lipophilic, [^{3}H]quinuclidinyl benzilate. Furthermore, it is also possible that the coupling of A2 and BK receptors to their respective G proteins could be differentially inhibited by PDBu. In fact it is not known whether the receptors for A2 and BK are coupled to different G proteins although in preliminary experiments we were unable to detect any effect of pertussis toxin (200 ng/mL; unpublished data).

Despite the apparent differences in sensitivity of BK all of the calcium-mobilizing agonists were inhibited by pre-incubation with PDBu. However, as reported previously [33], the response to ATP apparently involves two different receptor populations (high and low affinity). The response to 100 μM ATP showed significant sensitivity to PDBu pre-treatment, but it is worthy of note that the calcium entry component, which is only apparent at higher concentrations of the agonist (1 mM), was unaffected. Therefore if the receptor recognition sites are targets for protein kinase C, the high and low P_2 receptors themselves would have to be differentially regulated.

$InsP_3$ mass measurements were attempted in order to elucidate further the mechanisms involved. Unfortunately, the main problem encountered was that it was not possible to measure consistently an

$InsP_3$ response mediated by A2. The most likely explanation for this would appear to be a lack of sensitivity of the method since measurement of BK and ATP (the maximal calcium responses to both of which are larger than to A2)-mediated changes in $InsP_3$ was reproducible (it was not possible however to measure BK-induced $InsP_3$ production at the concentration at which calcium responses to A2 and BK were matched, a further indication of the lack of sensitivity of the method). Both agonists showed sensitivity to pre-treatment with PDBu thereby indicating that the site of protein kinase C is *proximal* to the $InsP_3$ receptor. The corresponding calcium response to BK (100 nM) was not sensitive to pre-treatment with PDBu; however, a significant (although quite modest) reduction in $InsP_3$ formation was observed suggesting that not all of the $InsP_3$ generated is necessary for a maximal calcium mobilization response. Comparison of ATP-mediated $InsP_3$ production and calcium mobilization however produced similarly sized, PDBu-mediated inhibitions. Thus, it would appear that the correlation between $InsP_3$ generation and calcium mobilization is not straightforward.

In summary, PDBu differentially inhibits calcium responses following stimulation with A2 and BK via activation of protein kinase C in populations of N1E-115 cells. The fact that this difference can be observed with matching calcium responses to the two agonists indicates that the possible targets for this differential effect are: (i) the receptor recognition sites, (ii) the coupling between the receptors and their respective G proteins or (iii) the involvement of different G proteins or isozymes of phospholipase C.

Acknowledgement—P.A.I. holds a SERC/CASE award with Boots Pharmaceuticals.

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