Protein kinase C stimulates PtdIns-4,5-P2-phospholipase C activity

Driss Radallah, Michel Nogaro, Bernard Fournier *

CNRS UPRESA 5017, Laboratoire de Physiopathologie et Pharmacologie Vasculaire, Université Bordeaux II Victor Segalen, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France

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Abstract

The tumour promoter, phorbol ester 12,13-dibutyrate (PDBu), acts on rectal palisadic epithelial cells and mimics the effects of neuroparsin, an antidiuretic neuronal hormone isolated from nervous lobes of the African locust corpora cardiaca. PDBu stimulated Ca\(^{2+}\)-dependent phospholipase C (PLC) activity resulting in inositol 1,4,5-trisphosphate (Ins\(^{1,4,5}\)P\(^3\)) production, increased cytosolic free calcium (monitored with the probe indo-1) and rectal fluid resorption. A 15-min pre-treatment with polymyxin B (PMXB), a protein kinase C (PKC) inhibitor acting at the phosphatidylserine (PS) binding site, suppressed PDBu stimulatory effects on free calcium entry and fluid resorption but not on phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-P\(^2\)) breakdown. On the contrary, bisindolylmaleimide Ro 32-0432 (which inhibits PKC at its ATP binding site) abolished entirely PDBu-stimulated PLC activity. It was concluded that two PKC are involved in transduction of the antidiuretic signal of neuroparsin. One PKC is PMXB sensitive and stimulates biological response after cytosolic free Ca\(^{2+}\) increase, while another PKC, insensitive to the PKC inhibitor, regulates the processes induced by the former PKC. Since PMXB-insensitive PKC exerts a stimulatory effect on PtdIns-4,5-P\(^2\)-PLC production, this original mechanism may be considered as a new signalling pathway under control of PKC. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Hormones or neurotransmitters which stimulate membrane effector PLC induce then PtdIns-4,5-P\(^2\) breakdown [1–3], resulting in production of two second messengers: Ins\(^{1,4,5}\)P\(^3\) which facilitates Ca\(^{2+}\) release from internal stores, and diacylglycerol (DAG) which activates phosphatidylinerine (PS) and Ca\(^{2+}\)-dependent PKC. PKC isoforms [4] catalyse in turn numerous protein phosphorylations which are essential signalling steps for cellular activation and subsequent biological responses.

The potent tumour promoters, phorbol esters,
stimulate PKC by substituting for DAG [5], but Unc-13 protein [6] and n-chimaerin [7] have been demonstrated to be phorbol ester receptors. A fundamental difference between phorbol esters and DAG has been reported [8,9]: DAG is quickly metabolised and activates PKC in a transitory fashion while phorbol esters are stable molecules which activate the enzyme over a longer time period.

In vertebrate cells, it has been described that either endogenous DAG or phorbol esters may modulate phosphoinositide breakdown by activating PKC [10,11]. PKC exerts negative feedback on PtdIns-4,5-P2 hydrolysis by phosphorylation of receptors or GTP-binding proteins. However, in invertebrate cell types, there are no precise data on regulation of phosphoinositide turnover by PKC.

In Insects, implication of PKC activity in different cellular signalling pathways has also been reported. As an example, in Aedes aegypti, the enzyme phosphorylates Cl³ channel proteins [12]. In African locust rectum, and especially in palisadic epithelial cells [13,14], stimulation of PKC is initiated by the antidiuretic signal of neuroparsin [15], a neuronal hormone extracted from storage lobes of the corpora cardiaca [16]. Two different inositol phosphate (InsP) cascades were then observed following phosphoinositide breakdown [17,18]. One pathway involves phosphatidylinositol (PtdIns) and phosphatidylinositol 4-monophosphate (PtdIns-4-P) hydrolysis under unknown PLC activity, whatever the concentration of neuroparsin. High concentrations of the agonist induce a second pathway which implicates pertussis toxin-insensitive heterotrimeric Goq protein, PtdIns-4,5-P2-PLC activity and complex InsP turnover. PKC plays a pivotal role by acting on (1) Ca²⁺ influx via the opening of L-type channels [14], (2) fluid resorption [15], and (3) soluble guanylyl cyclase activity [19,20] which, finally, down-regulates Ca²⁺ influx.

Another originality observed after PKC stimulation by DAG analogues and phorbol esters, is an increase in PLC activity, as neuroparsin does [13–15,18]. However, the different steps of such a pathway still remain unknown since previous experiments performed using Dowex columns did not allow to separate InsP isomers.

In this study, we analysed the PKC roles exhibited in both pathways under control of antidiuretic signal from neuroparsin. We demonstrated a new function for one PKC, which induced or potentiated PtdIns-4,5-P2-PLC activity. Investigations were carried out using PDBu as PKC activator and PMXB as inhibitor, a suitable biological assay to measure rectal fluid resorption, microspectrofluorimetry (and indolo-1 probe) to analyse cytosolic free Ca²⁺ movements, anion-exchange HPLC to separate InsP isomers, and binding experiments.

2. Materials and methods

2.1. Insects

We used 15–20-day-old male Locusta migratoria migratorioides, reared in the laboratory at 30°C under crowded conditions, and fed with fresh wheat and bran [13–20].

2.2. Hormone and chemicals

Neuroparsin was extracted from storage lobes of the corpora cardiaca or obtained by molecular biology tools [15,16], then purified in the laboratory. Maximal fluid resorption was generated with a 0.5 gland pair equivalent (0.5 cc), which corresponds to a concentration of agonist equivalent to 2 × 10⁻⁷ M.

Chemicals were purchased from Sigma (St. Louis, MO, USA): EGTA, EDTA, inositol 1-monophosphate (InsP₁), inositol 1,4-bisphosphate (InsP₂), InsP₃, inositol 1,3,4,5-tetrakisphosphate (InsP₄), PKC activators (as 1-stearoyl-arachidonoyl-sn-glycerol (SAG), or phorbol-12-myristate-13-acetate (PMA), or PDBu, PS, and PMXB sulfate, which inhibits PKC at its regulatory site by competition with PS [21,22]. Another PKC inhibitor, Ro 32-0432, which acts at the ATP binding site, was purchased from Alexis Biochemicals (Roger, Paris, France). The different substances were prepared as stock solutions by dilution in either physiological medium, or in 0.03% methanol (PDBu, PMA), or in 0.1% dimethylsulfoxide (SAG, Ro 32-0432), before suitable dilutions in medium.

d-\text{myo}-[2\text{-}^{3}\text{H}]\text{inositol (10–20 Ci/mmol)}, [\text{H}]\text{inositol polypophosphate marker set (containing InsP₁, InsP₂ and InsP₃), InsP₄, InsP₆, d-}
myo-[2-3H]Ins(1,3,4,5)P4; 20-60 Ci/mmoll were purchased from the Radiochemical Centre (Amersham, UK), while myo-inositol-2-3H(N)InsP6 (10-30 Ci/mmoll) was purchased from NEN (DuPont de Nemours, Les Ulis, France).

2.3. In vitro rectal sac preparations

Antidiuretic activity of different substances was measured using the biological assay of everted sac preparations [15]. Briefly, following a 1-h equilibration in chloride-free saline (25°C) derived from the normal saline of Hanrahan, oxygenated with 95% O2/5% CO2, rectae were filled with 10 μl of the normal saline complemented with neuroparsin or chemicals, or not (controls). Organs were incubated for an additional hour in normal oxygenated medium. Fluid resorption was measured as μl/sac per h for each experimental group, and data were then expressed as percentages, as compared to controls.

PMXB treatments were performed for 15 min, as described [14,18]. After washing, rectae were filled with 10 μl of normal saline complemented with neuroparsin or chemicals, or not (controls). Osmotic pressures of salines containing the different drugs were measured prior to all experiments, and were equivalent to that of standard saline (425-450 mosmol).

2.4. Cytosolic free calcium measurements

After removing and washing, isolated rectae were cut along their length and placed on rubber disks (2.5 cm diameter, 0.5 cm height) having a circular hole (7 mm diameter) in their centre and fixed with small needles, the haemolymph side above. A small PVC cylinder (0.5 cm diameter, 0.6 cm height) was then placed on each organ and was introduced into the hole by gentle pressure while needles were progressively exited. In these conditions, the luminal side of the rectum was placed toward the bottom of the disk and maintained closed by a glass coverslide which was fixed with silicone grease into a perforated cell culture box (3 cm diameter), as reported [14]. To prevent drying, 50 μl of standard saline were added into the central cavity of the disk delimited by the PVC cylinder and locust tissues, while 1 ml of the saline was placed around the disk.

Incorporation of the calcium probe indo-1 was performed as described [14]. Briefly, the saline was changed for similar saline containing 5 μM indo-1 penta-acetoxymethyl ester (indo-1/AM, Calbiochem, San Diego, CA, USA) and complemented with 0.02% Pluronic F-127 (Molecular Probes, USA). Rectae were oxygenated and incubated for 30 min at 20 ± 1°C. Between two successive stimulations, organs were washed with standard saline, and the apparatus was filled with 50 μl standard saline before fluorescence measurements.

Microspectrofluorimetry was carried out at 25°C and calcium concentrations were routinely estimated from indo-1 fluorescence by the ratio method using single wavelength excitation (355 nm) and dual emission at 405 nm and 480 nm. Our experiments being achieved on tissues and not on cultured cells, only changes in the ratio $F_{405}/F_{480}$ ($R$) were recorded, as discussed [14]. Results were expressed as percentages of changes in fluorescence intensities as measured in controls. All conditions and limits (calibration curve, viabiliy of tissues, etc.) were similar to those previously reported. At least three or four similar experiments were performed for either stimulation or control conditions.

2.5. Labelling of inositol lipids

The protocol of everted rectal sac preparations was used for the phosphoinositide labelling. Rectae were filled with 10 μl of Hanrahan’s saline containing 0.2 μCi of myo-[2-3H]inositol and incubated at 25°C for 1.5 h. At the end of this period, rectae were emptied and incubated again with 10 μl of the same labelled solution for an additional 1.5-h incubation, since the proportion of the radioactivity expressed by inositol lipid fraction, as compared to the total radioactivity, became stable after the third hour of incubation [18]. This protocol of inositol lipid labelling was used before measurements of PLC activity or anion-exchange HPLC separation of InsP isomers.

2.6. Phospholipase C assay

At the end of the labelling period, tissues were homogenised [23,24] with a 8 ml Kontes potter in 4 ml Bistris (20 mM, pH 6.5), 0.1 mM EDTA and
were stopped by sonicating rectae (pools of 6–8 organs) in 1.5 ml of a solution containing 5 mM KH$_2$PO$_4$, 10 mM EDTA (pH 6.3), 0.2 µg each of myo-inositol, mannitol, phytic acid, and each of the unlabelled Ins$_{1}$P$_{1}$, Ins$_{1,4}$P$_{2}$, Ins$_{1,4,5}$P$_{3}$ and Ins$_{1,3,4,5}$P$_{4}$ as carriers [25]. Then samples were centrifuged at 10000 × g for 5 min.

InsP isomer separation was performed on Beckman Gold HPLC system using Partisil 10-SAX Interchrom anion-exchange column (25 × 0.46 cm; 10 µM; from Interchim, Montluçon, France), as described [18], and by increasing concentrations in ammonium formate (pH 3.7) from 0 to 3 M, under constant flow rate (1.1 ml/min). To achieve a good separation of InsP isomers, 0.44 ml fractions were collected. Standards were separated in the following order: inositol, glycerophosphorylinositols (GPI), Ins$_{1}$P$_{1}$, Ins$_{1,4}$P$_{2}$, Ins$_{1,3,4}$P$_{3}$, Ins$_{1,4,5}$P$_{3}$ and Ins$_{1,3,4,5}$P$_{4}$. All these substances were detected in locust rectal tissues as well as two other InsPs previously identified as putative inositol 4-monophosphate (Ins$_{3}$P$_{1}$) and inositol 1,3-bisphosphate (Ins$_{1,3}$P$_{2}$). However, very low recovery of the radioactivity of standards was recorded after elution under high concentrations of the mobile phase. To overcome the problem, fractions corresponding to low concentrations of elution buffer were diluted in 1 ml water while fractions corresponding to high concentrations were diluted in 4 ml water [18]. Then, 10 ml of scintillation liquid were added to the samples before counting. Such modifications permitted to recover about 70% of the known radioactivity.

Since stimulation of neuroparsin-induced phosphoinositide system did not affect GPI production (i.e., had no effect on PLA activities), the ratio GPI/inositol remained constant and values calculated for each sample aligned perfectly (cf. Pearson’s test) on a straight line, according to a function $Y = aX + b$ (where $Y$ represents GPI concentration and $X$, inositol level). To correct the variability in size of the rectal tissues, we expressed the quantity of radioactivity corresponding to each InsP separated using the following equation [18,25]:

$$Q_{\text{InsP}} = \frac{\text{dpm} \text{InsP}}{\text{dpm} \text{GPI}} K_{\text{GPI}}$$

($K_{\text{GPI}} = 530$, or arbitrary constant value of GPI; *original radioactivity value).
2.8. \(^{3}H\)PDBu binding analysis

Rectae were homogenised in 10 ml of 10 mM Hepes (pH 7.3) containing 5 mM MgCl\(_2\), 1 mM EGTA and protease inhibitors: 1 mM PMSF, 2 µg/ml aprotinin, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin [26]. Membranes (700 µg/ml) were ultra-centrifuged at 30 000 \(\times\) g and at 4°C for 30 min (Ti45 rotor, Beckman). The pellets were re-suspended in 200 µl 10 mM Hepes and aliquotted (24 µg/sample), then pre-incubated (25°C) for 15 min with 1000 UI (Sigma) PMXB. \(^{3}H\)PDBu (NEN) binding experiments were performed with 5 nM of the PKC activator, and in the presence of 0.1 mM Ca\(^{2+}\). \(^{3}H\)PDBu was used on either untreated membranes or PMXB-treated membranes (in these conditions, PMXB was kept in the incubation medium). Free and bound radioactivities were separated by 8-min centrifugation at 20000 \(\times\) g.

2.9. Statistical expression of results

Standard errors were shown in graphs. The student \(t\)-test was performed to compare control (Ct) and experimental values: \(*P < 0.05\), \(**P < 0.01\), or \(***P < 0.005\), respectively, as compared to controls.

![Fig. 1. Changes in fluid resorption, measured on everted rectal sac preparations, after a 15-min pre-treatment with different concentrations of PMXB, and 1 h stimulation by 2 \(\times\) \(10^{-7}\) M neuroparsin (NP), or not (controls). Mean \(\pm\) S.E. (n = 6–8); significant results compared to controls (Ct): \(*P < 0.05\), \(**P < 0.01\), \(***P < 0.005\).

![Fig. 2. Effects of different PKC activators on fluid resorption. (A) Rectae were stimulated by either 5 \(\times\) \(10^{-6}\) M SAG, 5 \(\times\) \(10^{-5}\) M PMA, or 10 \(\times\) \(10^{-6}\) M PDBu, after a 15-min pre-treatment with 1000 UI Sigma PMXB (dashed columns), or not (white columns). (B) Dose-dependent effects of increasing concentrations of PDBu on biological response. Mean \(\pm\) S.E. (n = 6–12); significant results compared to controls (Ct): \(*P < 0.05\), \(**P < 0.01\), \(***P < 0.005\).

3. Results

3.1. Effect of PMXB on neuroparsin-induced fluid resorption

After a 1-h incubation period, 0.5 cc neuroparsin increased fluid resorption by 100% while 15 min pre-treatment with 1000 UI (Sigma) PMXB used alone increased the fluid resorption by 20%, compared to controls (Fig. 1). When neuroparsin was used after PMXB pre-treatment, the stimulatory effect of the agonist was suppressed. A dose-response curve was achieved with increasing concentrations of PMXB (Fig. 1B). Since maximum inhibition was recorded with 1000 UI (Sigma), the concentration was then used in experiments.
3.2. Effects of PKC activators on fluid resorption

Similar stimulation of fluid resorption as did the agonist, was induced by either $5 \times 10^{-6}$ M SAG, $5 \times 10^{-5}$ M PMA, or $10^{-6}$ M PDBu; but PDBu remained the most efficient PKC activator, able to act at the lowest concentrations (Fig. 2A). We also demonstrated that enhancement in biological response depended exclusively on PKC activity since PMXB abolished effects of activators. A dose–response curve was obtained with increasing concentrations of PDBu. Maximum effect occurred with 1 μM PDBu (Fig. 2B), while a plateau phase was detected in the presence of higher PDBu concentrations, up to 100 μM.

Similar biological responses were induced in the presence of either 1 μM PDBu or 80 μg/ml PS, or both drugs used simultaneously (Fig. 3A). On the contrary, fluid resorption dramatically decreased (Fig. 3B) in the absence of Ca$^{2+}$, i.e., after chelation with 1.2 mM EGTA.

A 15-min pre-treatment with 1000 UI (Sigma) PMXB (Fig. 3C) or 50 nM Ro 32-0432 (Fig. 3D) inhibited the biological response, whatever the drug used for PKC stimulation, i.e., in the presence of either 1 μM PDBu, or 80 μg/ml PS, or 1 μM PDBu plus 80 μg/ml PS used together. It was also observed that Ro 32-0432 did not enhanced fluid resorption when used alone.

3.3. Effects of PDBu on PLC activity

After a 10-min incubation, increasing concentrations of PDBu enhanced PLC activity in microsomes (Fig. 4A). The dose–response curve obtained with different PDBu concentrations resembles curve ob-

Fig. 3. Changes in fluid resorption after a 1-h stimulation with either 1 μM PDBu or 80 μg/ml PS, or PDBu+PS. Drugs were used alone (A), with 1.2 mM EGTA (B), or after a 15-min pre-treatment with either 1000 UI Sigma PMXB (C) or 10 nM Ro 32-0432 (D). Mean ± S.E. ($n=7-9$); significant results compared to controls (Ct): *$P<0.05$, **$P<0.01$. 
tained for fluid resorption in similar conditions. Superimposition of 80 mg/ml PS treatment to 1 μM PDBu treatment potentiated weakly but significantly PLC activity stimulated by PDBu.

PLC activity significantly increased in the presence of 1 μM PDBu and 0.1 mM Ca$^{2+}$ (Fig. 4B). On the contrary, the divalent cation chelator EGTA provoked a drop in PDBu effect, although the latter still remained positive and significant, as compared to controls.

A 15-min pre-treatment with PMXB did not abolish the stimulatory effects of 1 μM PDBu exhibited in the presence of 80 μg/ml PS, or not (Fig. 4C). In these conditions, PDBu effect surprisingly appeared different to that obtained for fluid resorption, under similar conditions (see Fig. 2A). On the contrary, a 15-min pre-treatment with Ro 32-0432 (50 nM) entirely abolished the stimulation of PLC activity induced by different chemicals (Fig. 4C).

### 3.4. Effects of PDBu on InsP production

InsP production increased in rectal cells when they were stimulated with increasing concentrations of PDBu (Fig. 5A), and a maximum was recorded with 1 μM PDBu. However, the increase in InsP production was only obtained after a minimum of 20 s treatment with 1 μM PDBu, but not for a shorter time (Fig. 5B). InsP production reached a maximum for 1 min PDBu stimulation. After InsP separation by anion-exchange HPLC, PDBu induced high levels in Ins$_{1;4}$P$_1$, Ins$_{4;1}$P$_1$, Ins$_{1;3}$P$_2$, Ins$_{1;4}$P$_2$, Ins$_{1;3;4}$P$_3$ and Ins$_{1;4;5}$P$_3$.

Addition of 80 μg/ml PS to 1 μM PDBu provoked earlier PDBu stimulatory effects (Fig. 5B): Ins$_{1;4;5}$P$_3$ production was obtained after a 5-s stimulation period. The highest level in InsP production was finally observed after 1 min incubation with PDBu plus PS.

PMXB pre-treatment changed the PDBu effects (Fig. 5B,E). Ins$_{1}$P$_1$, Ins$_{3}$P$_1$, Ins$_{1;4}$P$_2$, Ins$_{1;3}$P$_2$ and Ins$_{1;4;5}$P$_3$ productions significantly increased after 20 s stimulation with 1 μM PDBu, while a longer incubation period (1 min) did not improve the result.

Furthermore, InsP production did not change after 15 min PMXB pre-treatment then 5 s PDBu (1 μM) stimulation, in the presence of 80 μM/ml PS (Fig. 5B,D,F): InsP production remained similar to that of controls (or PDBu when used alone). On the contrary, after a PMXB treatment (Fig. 5F), a longer stimulation (20 s) with both PDBu and PS resulted in InsP production similar to that induced in the absence of PMXB treatment. After a 1-min
Fig. 5. Separation of InsPs isomers by anion-exchange HPLC. (A) Total InsP production after stimulation with increasing concentrations of PDBu, used alone (white columns) or in the presence of PS (dashed columns). (B) Total InsP production with either PDBu or PDBu+PS, for different times, and after PMXB pre-treatment (dashed columns), or not (white columns). (C,D) InsP productions for different times after stimulation with PDBu or PDBu+PS, respectively. (E,F) InsPs produced in similar conditions as in C and D, respectively, but after PMXB pre-treatment. Concentrations of PDBu, PS and PMXB, when used alone, were 1 μM, 80 μg/ml, and 1000 UI, respectively. Mean ± S.E. (n = 5–10).
stimulation in similar conditions, $\text{Ins}_{1,3}P2$ and $\text{Ins}_{1,4,5}P3$ productions further increased.

3.5. Effects of PDBu on cytosolic free calcium movements

Calcium movements were measured in both categories (palisadic and basal) of epithelial cells. In palisadic cells (Fig. 6A), 1 mM PDBu induced a rise in $R$ reflecting $[\text{Ca}^{2+}]_i$, after a 5-s delay (Fig. 6A-1). This approximately reached 20%, as compared to basal level. A return to basal level was recorded after 35 s. Furthermore, a treatment performed with the L-type $\text{Ca}^{2+}$ channel blocker nifedipine (Fig. 6A-2) provoked a decrease in $R$. In these conditions, 1 μM PDBu became not efficient to induce elevation in $R$. In other hand, and after 15 min PMXB treatment (Fig. 6A-3), PDBu (1 μM) effect on $R$ in palisadic cells was null, i.e., similar to that of controls.

In rectal basal cells (Fig. 6B), 1 μM PDBu induced a slight increase in $R$ after a 26-s delay (Fig. 6B-1). Then $R$ returned to the basal level. This effect be-

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![Fig. 6](image_url)

**Fig. 6.** Measurement of $R$ reflecting cytosol free $\text{Ca}^{2+}$ concentration in living epithelial rectal cells. The different substances were added only when a stable horizontal line was attained for the $R$ record. (A) Stimulation of $R$ palisadic cells with 1 mM PDBu (1), in the presence of 1 μM of the L-type $\text{Ca}^{2+}$ channel inhibitor nifedipine (Nif) or not (2), and after pre-treatment with 1000 UI PMXB (P) (3). (B) Similar experiments performed in basal cells; trace 4 corresponds to trace 1, while traces 5 and 6 correspond to traces 2 and 3, respectively.
came null in the presence of nifedipine (Fig. 6B-2) or after a pre-treatment with the PKC inhibitor PMXB (Fig. 6B-3).

3.6. PDBu binding after PMXB treatment

[^3]H]PDBu binding increased upon a very short incubation period, then it reached a plateau after 10 min. A 15-min treatment with the PKC inhibitor PMXB provoked a large decrease in[^3]H]PDBu binding to microsomes, over a short time incubation period (Fig. 7). A longer incubation (10 min) re-established similar[^3]H]PDBu binding as observed in stimulated controls, i.e., in the absence of PMXB. However, when incubation time increased to 30 min,[^3]H]PDBu binding in PMXB-treated microsomes decreased again to an intermediate value comprised between that recorded at the beginning of the experiment and that obtained for a 10-min incubation period.

4. Discussion

This study shows that PDBu, a known potent PKC activator [5,9], increased (1) cytosolic free Ca^{2+} levels in palisadic epithelial cells of the African locust rectum and (2) fluid resorption, as did other PKC activators (PMA or SAG, a DAG analogue) or the antidiuretic neuronal hormone neuroparsin [13–15,17]. Basal epithelial cells of the rectum were weakly sensitive to PDBu effect while the substance had no effect on other rectal cells [14]. Furthermore, PMXB pre-treatment abolished PDBu-induced stimulation of Ca^{2+} influx and biological response. Thus, the data may suggest the involvement of PKC in both processes, as phorbol ester receptors.

We have reported that neuroparsin, as a function of its concentration, rapidly (5 s) mobilises two different phosphoinositide pathways in palisadic epithelial cells of the locust rectum through involvement of two different Ca^{2+}-dependent PLC activities [18]. One pathway starts from PLC activity which hydrolys low phosphorylated substrates (PtdIns or PtdIns-4-P) and produces Ins_{1,4,5}P_{3}, or Ins_{1,4}P_{2} then Ins_{4,5},P_{1}, respectively, whatever the concentration of neuroparsin. High concentrations of the agonist activate PtdIns-4,5-P_{2}-PLC and Ins_{1,4,5}P_{3} production. Both PLC activities induced by neuroparsin are inhibited after a 15-min PMXB treatment [13,18].

In this study, we demonstrated that PDBu also stimulated Ca^{2+}-dependent PLC activity and increased an early (5 s) Ins_{1,4,5}P_{3} production, and the phospholipid PS accelerated and enhanced PDBu effect (Fig. 5). However, a major difference exists between effects of PDBu and neuroparsin since PDBu did not stimulate an early (5–20 s) Ins_{1,4}P_{1} production and because Ins_{1,4}P_{2} and Ins_{4,5}P_{1} significantly increased from 20 s to 1 min of incubation. PtdIns/PtdIns-4-P-PLC activity being not efficient before 20 s, data indicate a preferential effect of PDBu on the phosphoinositide pathway implicating PtdIns-4,5-P_{2}-PLC activity (i.e., Ins_{1,4,5}P_{3} production at 5 s). Production of Ins_{1,4}P_{2} and Ins_{4,5}P_{1} observed at 20 s without any change in Ins_{1,4}P_{1} corresponds to the effects of monophosphatases which hydrolysed Ins_{1,4,5}P_{3}, Ins_{1,3,4,5}P_{4}, and metabolites (as Ins_{1,3,4}P_{3}, for example) rather than the result of specific PtdIns-4-P-PLC activity. On other hand, PDBu still remains able to stimulate high PtdIns-4,5,P_{2}-PLC activity after PMXB treatment, and low PtdIns/PtdIns-4-P-PLC activity. Thus, we can definitely assume the existence of two phosphoinositide pathways in palisadic cells of locust rectal epithelium, each mobilising different PLC and ‘PKC’ activities. It is obvious that the biological response is under the control of a PMXB-sensitive ‘PKC’ since we demonstrated that PMXB inhibited PtdIns/PtdIns-4-P hydrolysis, cytosolic free Ca^{2+} increase.
The PMXB-insensitive 'PKC' is directly implicated in positive feedback on PtdIns-4,5-P2-PLC activity. Our postulate for such a new pathway generates at least two comments. Firstly, both 'PKC' activities were independent each other since PMXB did not suppress PDBu-stimulated PtdIns-4,5-P2-PLC activity, i.e., production of Ins$_{1,4,5}$P3 (Fig. 5). Whatever the PDBu concentration, we did not record an early increase in Ins$_{1}$P1 and Ins$_{4}$P1 productions, which is interpreted as a blockage of basal PtdIns/PtdIns-4-P breakdown. Thus, neuroparsin-induced stimulation of each 'PKC' suggests either the involvement of a chronology in DAG binding to each 'PKC', or the participation of two DAG having different molecular structures (Carricaburu and Fournier, unpublished results) to avoid confusion in transduction of specific antidiuretic messages expressed by the agonist. On the contrary, since PMXB totally suppressed the early effects of neuroparsin on both phosphoinositide breakdowns, it may be supposed that other enzymes than 'PKC' (and independent of this enzyme) are mobilised by the agonist. This is not the case with PDBu, which specifically acts on 'PKC', and not through receptor-G protein-PLC complex, i.e., when the integrity of phosphoinositide pathway was preserved. In addition, this may suggest that neuroparsin has probably one rather than two different receptors, or requires an entire mobilisation of the first pathway to act then on the second pathway, via receptor and G protein.

An original finding is the ability of the second 'PKC' (whose activity results from PtdIns-4,5-P2 breakdown) to stimulate the PLC which hydrolyses PtdIns-4,5-P2 substrate. It has been described that phorbol esters decrease InsP production in vertebrate cells by either phosphorylation of receptors or GTP-binding protein involvement [8,9], while stimulation of phosphoinositide breakdown after a PDBu treatment is rare [27,28]. Thus, such ability for 'PKC' to stimulate PtdIns-4,5-P2 breakdown must be considered as an original process.

On other hand, a surprising result was obtained after PMXB pre-treatment. The fluid resorption enhanced when the drug was used alone, while the PKC inhibitor was assumed to suppress the early PtdIns/PtdIns-4-P-PLC activity, Ca$^{2+}$ entry, and biological response induced by either neuroparsin or PDBu. As a first hypothesis, we observed after 10 min of incubation in the presence of $[^3H]$PDBu (Fig. 7) that PMXB-treated membranes exhibited similar $[^3H]$PDBu binding to that showed by PMXB-untreated membranes. This may result from possible uncoupling of PMXB from 'PKC' while PS took its place, since it was reported that PMXB competes with PS for binding to the regulatory site of PKC [29]. Thus, 'PKC' inhibition being lifted, the integrity in both phosphoinositide pathways was restored and, even in the presence of PMXB, some sporadic Ca$^{2+}$ influx became possible as well as an enhancement in PtdIns/PtdIns-4-P-PLC activity, which produced lowly phosphorylated InsPs, as Ins$_{1}$P1, or Ins$_{1,4}$P2 and Ins$_{4}$P1 (Fig. 5), and also the stimulation of fluid resorption. As a second hypothesis, it may be supposed the involvement of a non-phosphoinositide pathway responsible for a negative feedback on cytosolic free Ca$^{2+}$ influx, via the participation of a soluble guanylyl cyclase [14,17,19]. We reported that antidiuretic peptides from glandular lobes of locust and cockroach corpora cardiaca are able to stimulate rectal fluid resorption, through cGMP- and cAMP-dependent kinases [19,20,30]. As another explanation, possible synergy between processes evoked in both hypotheses may be also retained to explain stimulation of fluid resorption in the presence of PMXB.

Another problem arises with treatment of locust rectal tissues with PMXB because high Ins$_{1,4,5}$P3 production (i.e., high PtdIns-4,5-P2-PLC activity) was maintained for a long time. PtdIns-4,5-P2 breakdown generally occurs early and rapidly under a neuroparsin effect [18]. In our experiments, Ins$_{1,4,5}$P3 was slowly metabolised into lowly phosphorylated InsPs. As an interpretation, PMXB-sensitive 'PKC' may control InsP turnover generated by PtdIns-4,5-P2 breakdown. This is another argument for the existence of one rather than two receptors to bind the neuroparsin.

Chimaerins (and Unc-13 protein) exhibit high affinity for phorbol esters, as PKC isozymes [6,7]. They have one single cysteine-rich region with homology to both present in PKC, and are GTPase activating proteins for members of the Rho subfamily of small GTP-binding proteins. A specificity of chimaerins is that they require higher concentrations in phospho-
lipid for PDBu binding than PKC, but despite these differences it appears difficult to distinguish between chimaerins and PKC in the absence of some knowledge in enzyme activation as well as in their respective targets. In our model, we demonstrated that the ATP site PKC inhibitor, bisindolylmaleimide Ro 32-0432, not only suppressed stimulatory PDBu effects on fluid resorption as did PMXB, but also abolished PDBu-induced stimulation of PtdIns-4,5-P2 hydrolysis, which was not the case with PMXB. Furthermore, we have stated that heterotrimeric Goq11 proteins and βII subtype of PtdIns-4,5-P2-PLC participated in induction of phosphoinositide pathway involving PMXB-insensitive ‘PKC’ (Radallah et al., personal communication). This PMXB-insensitive ‘PKC’ regulated in a classical manner the Ca2+ influx induced by the PMXB-sensitive ‘PKC’ involved in the first phosphoinositide system, via GMPc enhancement [20]. Finally, both phorbol ester receptors present in our model have been identified as members of the phospholipid and Ca2+-dependent PKC family, using different techniques of chromatography, autoradiography and immunological tools. All these arguments represent some positive probes that phorbol ester receptors involved in epithelial cells of the locust rectum are PKC rather than chimaerins.

In conclusion, two PKC are present in palisadic epithelial cells of the locust rectum. It becomes possible now to dissociate the activity of each PKC using efficient pharmacological tools. One major pathway involves a PKC able to stimulate the PtdIns-4,5-P2-PLC which indirectly activates it, and exerts negative feedback on free-Ca2+ influx, dependent on PMXB-sensitive PKC, and responsible for fluid resorption. Such an original pathway may be considered as a specific adaptation for regulation of the biological response, fluid resorption.

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References