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Phorbol myristate acetate induces endocytosis as well as exocytosis and hydroosmosis in toad urinary bladder

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The induction of the hydroosmotic response in the toad urinary bladder is considered to be associated with membrane addition mediated by exocytosis at the affected luminal membrane and reversed by endocytic retrieval at that surface. The permeability, exocytosis and endocytosis are initiated by antidiuretic hormone (ADH) receptor interaction on the basolateral membrane. In other hormone responsive systems, phorbol ester (phorbol myristate acetate, PMA), a tumor promoter, has been implicated in the regulation of various transport processes through the activation of protein kinase C and cytoskeletal protein phosphorylation. We found that addition of 10^{-6} M PMA to the mucosa induces an hydroosmotic response which is gradual and which reaches a maximum within 60 min, equal to about 1/3 the maximal ADH response. Morphologically, PMA causes rapid exocytosis of the granules, endocytosis of horseradish peroxidase from the mucosal medium into tubules and multivesicular bodies and elongation of apical microvilli. Controls treated with mucosal 0.1% dimethylsulfoxide (DMSO) or an inactive PMA isomer on the mucosal surface, or PMA on the serosal surface lack the hydroosmotic, exocytic, endocytic and cytoskeletal changes. Addition of serosal ADH to PMA-treated bladders results in a precocious hydroosmotic and exocytic ADH response, but a lowering of the maximal response. Also pretreatment of bladders with PMA prevented the ADH-induced increase in transepithelial potential difference. Thus, apical events mediating the PMA hydroosmotic response are correlated with exo- and endocytosis and elongation of apical microvilli.

Introduction

Phorbol myristate acetate (PMA), a tumor promoter, has been shown to induce exocytosis in several cell types [1,2]. In our previous studies, we had correlated the effect of the antidiuretic hormone (ADH) on hydroosmosis in the toad urinary bladder with exocytosis and endocytosis [3,4]: exocytic addition of membrane to the apical surface is initiated at the same time as the hormonally induced increase in hydroosmosis across the apical region [5]. Subsequent endocytic retrieval and internalization of membrane from the apical surface parallels a diminution in the net water flux in the presence of ADH [4,5]. ADH activates adenylate cyclase [6], elevates the level of the intracellular messenger, cyclic adenosine monophosphate, cyclic AMP (cAMP), and initiates apical membrane cycling and hydroosmosis [3,4]. Since ADH can also stimulate phos-

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

phoinositide turnover [7] and thereby activate protein kinase C, it is possible that some element of the hormone response could be mediated through this pathway. The present study was undertaken to test whether PMA, a direct activator of protein kinase C could affect the physiologic state of the toad bladder, and to determine whether this is accompanied by an induced membrane cycling. Of special interest were: (1) could PMA initiate exocytosis as it does in other systems, (2) could PMA initiate endocytosis as ADH does, and (3) could mucosal application of PMA duplicate ADH effects. Previously, all agents reported to initiate the hydroosmotic response and exo- and endocytosis act on the basal-lateral (serosal) side of the epithelial cell, while the characteristic effect is produced on the apical side as a result of the intracellular production of second messenger(s).

We report that addition of PMA to mucosal solution promotes endocytosis as well as exocytosis and hydroosmosis but does not fully mimic ADH. The relative magnitude and timing of hydroosmosis and exocytosis are different from that induced by ADH. Furthermore, when PMA treatment precedes ADH challenge, the maximal ADH-induced hydroosmosis is more rapidly achieved. Morphologically, PMA either alone or with ADH produces a dramatic elongation of microvilli. Serosal addition of PMA had none of these effects. Thus, a fusogen (PMA) applied to the mucosal side does induce endocytosis, exocytosis and some hydroosmosis but in a pattern not identical with the complete ADH-induced hydroosmotic response.

Materials and Methods

Materials. 4β-Phorbol-12-myristate 13-acetate (PMA) (also known as 12-*O*-tetradecanoylphorbol 13-acetate, TPA), its inactive isomer, 4α-phorbol 12,13-didecanoate (αPDD), dimethylsulfoxide (DMSO, Fisher), horseradish peroxidase, (type II), 3,3′-diaminobenzidine tetrachloride (DAB), Hepes and Tris were purchased from Sigma Chemical (St. Louis, MO); antidiuretic hormone (ADH, Pitressin) from Parke, Davis (Morris Plains, NJ) and Ruthenium red from Polysciences (Warrington, PA). All other chemicals were reagent grade.

Hepes-buffered saline was used for all in vitro bladder incubations: 110 mm NaCl, 10 mm Hepes, 3 mM $KHCO_3$, 1 mM Na_2HPO_3, 5 mM dextrose, 1 mM $CaCl_2$, 1 mM $MgCl_2$ (pH 7.6), 230 mosmol/kg.

Physiological preparation. Urinary bladders were excised from doubly pithed, female, Dominican toads, *Bufo marinus* (National Reagents, Bridgeport, CT), and each hemibladder was mounted on a tubular assembly through which it was filled and emptied and on which the net weight loss was measured [8]. Bladders were filled and emptied, twice, and then refilled with 5 ml of full-strength Hepes-buffered saline and placed in full-strength Hepes-buffered saline for a preincubation period of up to 1 h. For the incubation, bladders were placed in a fresh Hepes-buffered saline bath and the mucosal solution replaced by dilute Hepes-buffered saline in which all constituents were diluted 1 : 4 with distilled water (fifth-strength Hepes-buffered saline, H/5). The hydroosmotic gradient thus produced across the tissue is a standard procedure used to approximate the salt gradient between urine and plasma [8]. To determine baseline hydroosmotic flux, i.e. net water loss, during a 30-min period, bladders were weighed at 10–15-min intervals. The challenge period was begun by adding PMA to the mucosal solution, dissolved in DMSO, (final concentration PMA 10^{-6} or 10^{-7} M, DMSO 0.1 or 0.01%). Paired control hemibladders mucosal solution contained 10^{-6} M αPDD in 0.1% DMSO, 0.1 or 0.01% DMSO alone. Net water movement, hydroosmosis was monitored at 10–15-min intervals and calculated from the rate of weight loss (mg/min). Transepithelial potential difference was measured potentiometrically (open circuit) at 15-min intervals after PMA, with or without ADH, in a group of representative bladders (minimum bladder pairs at each time-point, $n = 3$). Aeration of the serosal solution throughout the experiment gently agitates the bladders and their mucosal and serosal solutions. Osmolality of all solutions was measured by a vapor pressure osmometer (Wescor, Logan, UT).

In some experiments with short PMA exposure, to maintain our standard osmotic gradient we added PMA or DMSO to the corresponding serosal solution. These bladders were not significantly different in any of the characteristics we were measuring.

In experiments in which endocytosis was studied, 0.3% horseradish peroxidase was added to the mucosal solution along with PMA and/or DMSO.

Electron microscopy. Three types of tissue-processing provided different kinds of morphological information:

(1) For structural studies, bladders were emptied and refilled with 2% glutaraldehyde in 0.1 M cacodylate (pH 7.2), while simultaneously immersed in the same fixative. The bottom cm of the bladder, cut into smaller pieces, was stored in glutaraldehyde solution (0°C) for a maximum of 7 days after which they were postfixed with reduced OsO_4, 1–2 h (0°C) [9].

(2) For demonstration of endocytosis, after horseradish peroxidase exposure and fixation for 1–2 h in glutaraldehyde as described above, the bladder pieces were rinsed overnight in 0.1 M cacodylate buffer (0°C), and incubated for the demonstration of horseradish peroxidase reaction product in 0.05 M Tris buffer with 1% H_2O_2 and 5% diaminobenzidine, DAB, (pH 7.6) followed by OsO_4 [10]. Since the peroxidase was present in the mucosal solution, its presence inside a membranous structure identifies communication at some time with the apical surface and the mucosal solution [11]. We use horseradish peroxidase in sequence with a second marker presented at fixation to demonstrate true endocytosis: the complete interiorization of horseradish peroxidase-containing structures. This endocytosis can be demonstrated by comparison of the number of the peroxidase-containing structures with the number which contain an extracellular marked added at the time of fixation as described below [12].

(3) For visualization of the extracellular space and thus membranes which remain connected with the surface, bladders were filled with and immersed in 0.05% Ruthenium red, 1.2% glutaraldehyde in 0.067 M cacodylate (pH 7.2) [9] and postfixed with 0.05% Ruthenium red, 1.67% OsO_4 and 0.067 M cacodylate buffer (Ruthenium red/OsO_4) (pH 7.2).

In all preparations, the pieces were dehydrated in a graded series of alcohols, embedded in Epon and then silver sections were cut and examined either unstained or after uranyl acetate and lead citrate staining [13,14] in a JEOL 100B electron microscope.

Quantitation of granules and horseradish peroxidase- or Ruthenium red-containing structures in a minimum of three bladder pairs was performed by counting the numbers of each in at least eight consecutive granule-rich cells in one section per hemibladder. Measurement of microvillar length was performed on micrographs from six pairs of bladders. All microscopy was done on coded specimens the identity of which was unknown to the microscopist.

Results

Addition of PMA (10^{-6} M) to the mucosal solution induces a hydroosmotic response in the toad bladder (Fig. 1). The maximum rate of hydroosmotic flux is 8.76 ± 3.18 (S.D.) mg/min ($n = 7$) and is achieved after 60–90 min (Fig. 1); this is compared to a baseline of 1.065 ± 0.71 (S.D.) for control hemibladders treated with 0.1% DMSO. Mucosal 10^{-7} M PMA induces less of an hydroosmotic response than 10^{-6} M: its maximal response is achieved at 90 min and is 4.59 ± 1.92 (S.D.) mg/min ($n = 8$). Mucosal PMA at 10^{-8} or 10^{-9} M produced no effect on basal flow compared to DMSO-control hemibladders over the 90-min test period ($n = 8$). Application of 10^{-7} M PMA to the serosal side of the bladder does not alter the basal rate weight loss.

Preincubation of the bladder with PMA affects the subsequent response to ADH, (Fig. 2). The ADH-induced hydroosmotic flow in both the

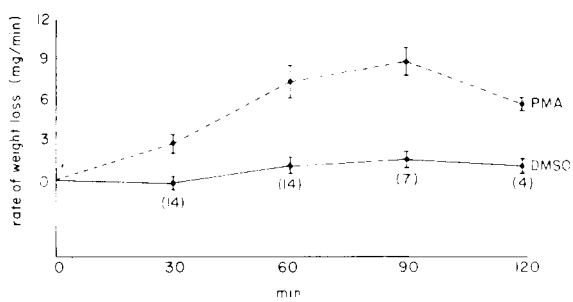


Fig. 1. Time-course of PMA-induced hydroosmosis. 10^{-6} M PMA in 0.1% DMSO added to the mucosal solution promotes a significant hydroosmotic response within 15 min. 0.1% DMSO does not affect the baseline. However, the maximal rate of PMA-induced weight loss achieved is significantly less than with ADH and DMSO (compare with Fig. 2a). The number of hemibladders is given in parentheses.

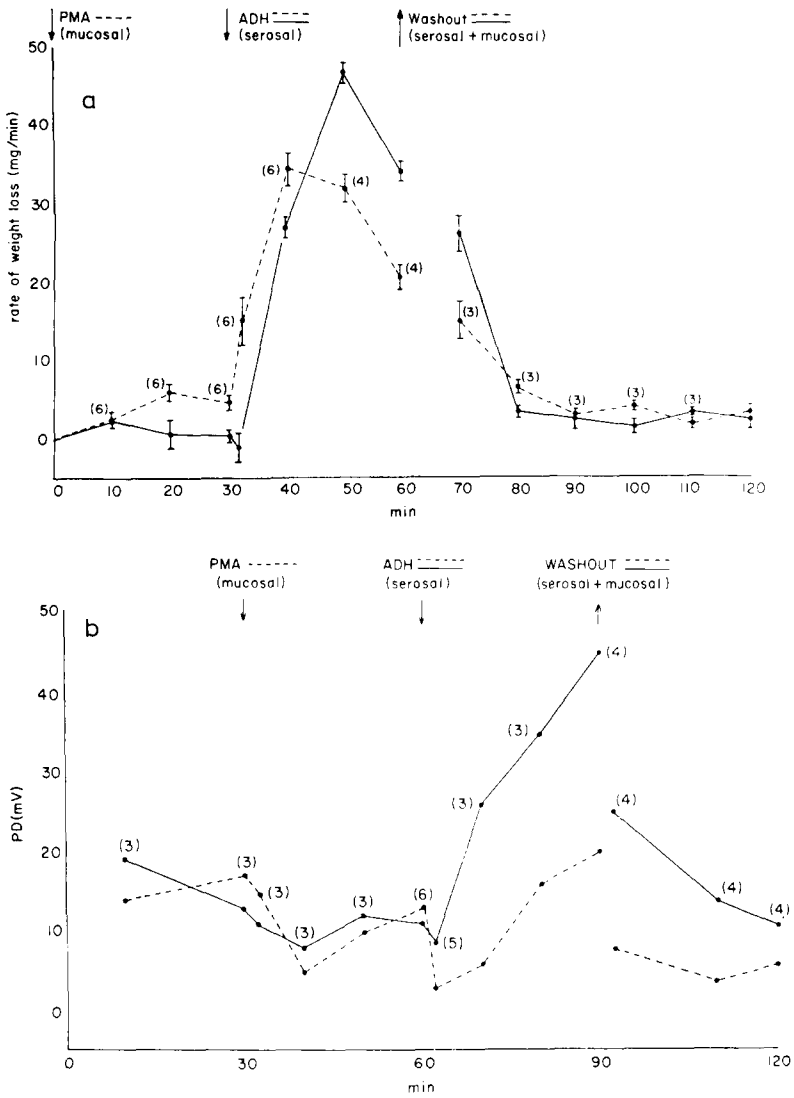


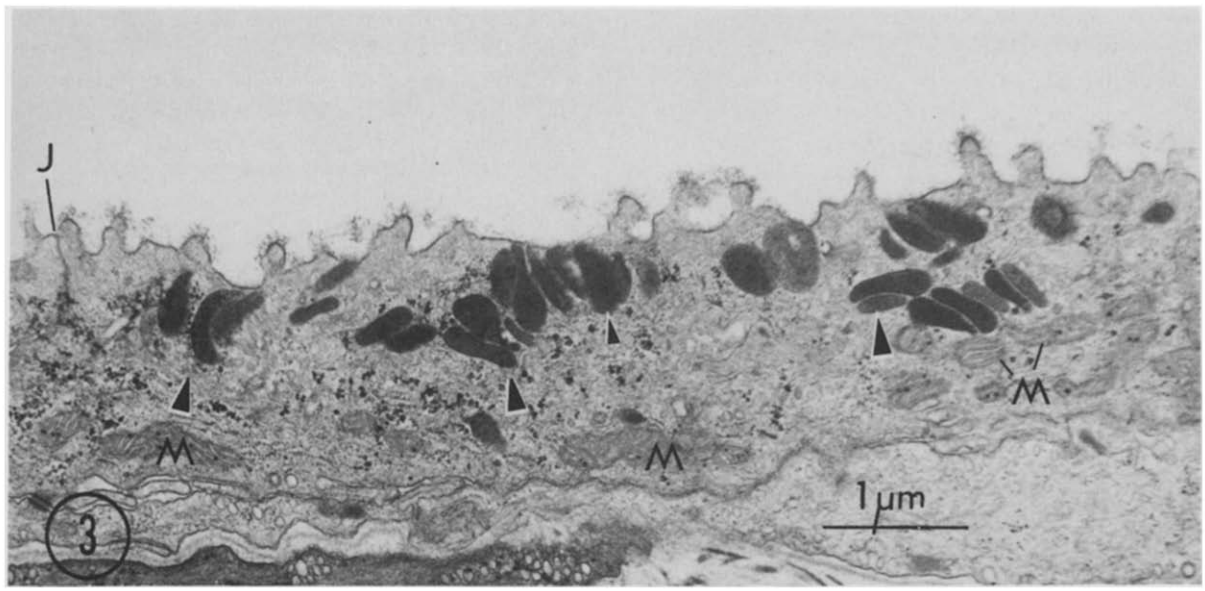
Fig. 2. Effect of pretreatment with mucosal 10^{-6} M PMA in 0.1% DMSO or 0.1% DMSO on serosal ADH-induced hydroosmosis (a) and transepithelial potential difference (b). The first arrow indicates addition of mucosal PMA (— — —) or DMSO (———), the second arrow indicates the addition of serosal ADH and the third arrow, washout of both mucosal and serosal solutions with Hepes-buffered saline. In the hydroosmotic response, PMA decreases the time to reach the maximal response as well as its magnitude. The ADH-induced transepithelial potential difference is inhibited by PMA. However, washout of ADH-induced permeability is not prevented by PMA.

PMA-pretreated and control hemibladders increased at the same initial rate; however, the PMA-treated hemibladders reached a maximum earlier. The maximal ADH-stimulated hydroosmotic rates in the two sets of hemibladders were quite different: DMSO and ADH (control) was 47.8 ± 3.95 (S.D.) mg/min at 20 min, and the PMA, DMSO and ADH (experimental) was 34.5 $\pm$ 8.83 (S.D.) mg/min (Fig. 2a) at 10 min. Thus, PMA pretreatment appears to prevent the full development of ADH-induced hydroosmosis. Similarly, the rate and maximum of ADH-induced transepithelial potential difference is diminished by mucosal PMA pretreatment (Fig. 2b).

Electron microscopy

Mucosal PMA produces dramatic changes in the apical region of both granule-rich and mitochondria-rich cells. Specifically, the granule-rich cells' apical microvilli are very elongated. In six experiments, the height of the three longest microvilli averaged 0.75 ± 0.06 (S.E.) μm after 10 min PMA treatment compared to 0.27 ± 0.04 (S.E.) μm in paired DMSO-treated control bladders (Fig. 3 vs. Figs. 4 and 5). These elongated apical microvilli are often seen to have highly oriented microfilaments (Fig. 6) not common in control bladders (Fig. 3).

PMA also induced exocytosis: the granule-rich



Figs. 3–8. Electron micrographs are of sections stained with uranyl acetate and lead citrate, except for Fig. 7 which is an unstained section, the bar on each micrograph represents 1 μm.

Fig. 3. Electron micrograph of portions of two adjacent granule-rich cells and their tight junction (J) in a control hemibladder exposed to the inactive isomer, αPDD 10^{-6} M in 0.1% DMSO. Granule rich cells of the toad urinary bladder have many membrane-bounded granules (arrowheads) in their apical region. Mitochondria are seen at M. The apical surface microvilli are generally low and undulating. ×20000.

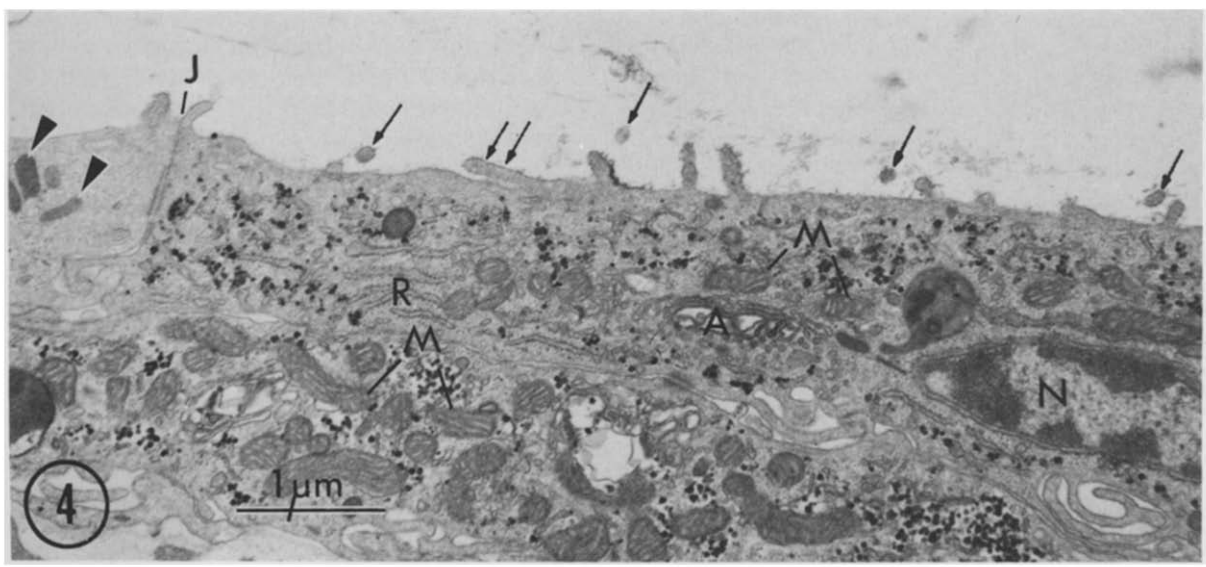


Fig. 4. Granule-rich cells in the paired experimental hemibladder after 10 min exposure to 10^{-6} M PMA in 0.1% DMSO. PMA causes elongation of the microvilli: this is easily seen when the microvillus is cut parallel to its long axis (double arrow) and can be inferred from the numbers of cross-sections of apparently isolated microvillar tips (single arrows) which are sufficiently distant from the basal region so they are not seen as a single entity. In that cell few, if any, granules are seen while the adjacent cell has a group of granules (arrowheads). The Golgi apparatus (A), rough endoplasmic reticulum (R), nucleus (N) and mitochondria (M) are also present. ×20000.

cells are generally devoid of granules at 10, 30 and 60 min of PMA exposure; control hemibladders have an average of 20–50 granules/thin section of cell (Fig. 3) (see also Ref. 5). Cell-to-cell variation is great during the early periods of PMA exposure so that at 5 or 10 min some cells are degranulated and have elongated microvilli (Fig. 5), an adjacent cell partially degranulated with a few elongated microvilli (Fig. 4), and some cells have many granules and gently rolling appearance of the apical microvilli similar to the controls. However, by 30 min the cells are uniformly degranulated and have elongated microvilli. This was also obvious at 60 and 90 min, the longest time-periods studied. The mucosal glycocalyx is often thicker after PMA, presumably contributed by exocytosis of their granule contents (Fig. 6). These early changes in granule content (exocytosis) and microvillus transformation are also seen in bladders treated with mucosal PMA in the absence of an osmotic gradient (not shown).

Endocytosis also is stimulated in the PMA-treated bladders. PMA stimulates mucosal horseradish peroxidase uptake into tubules, vesicles and multivesicular bodies of the granule-rich cells (Fig. 7): most cells have taken up horseradish peroxidase at 15 min of PMA exposure, but by 30 min, all granule-rich cells have some peroxidase-

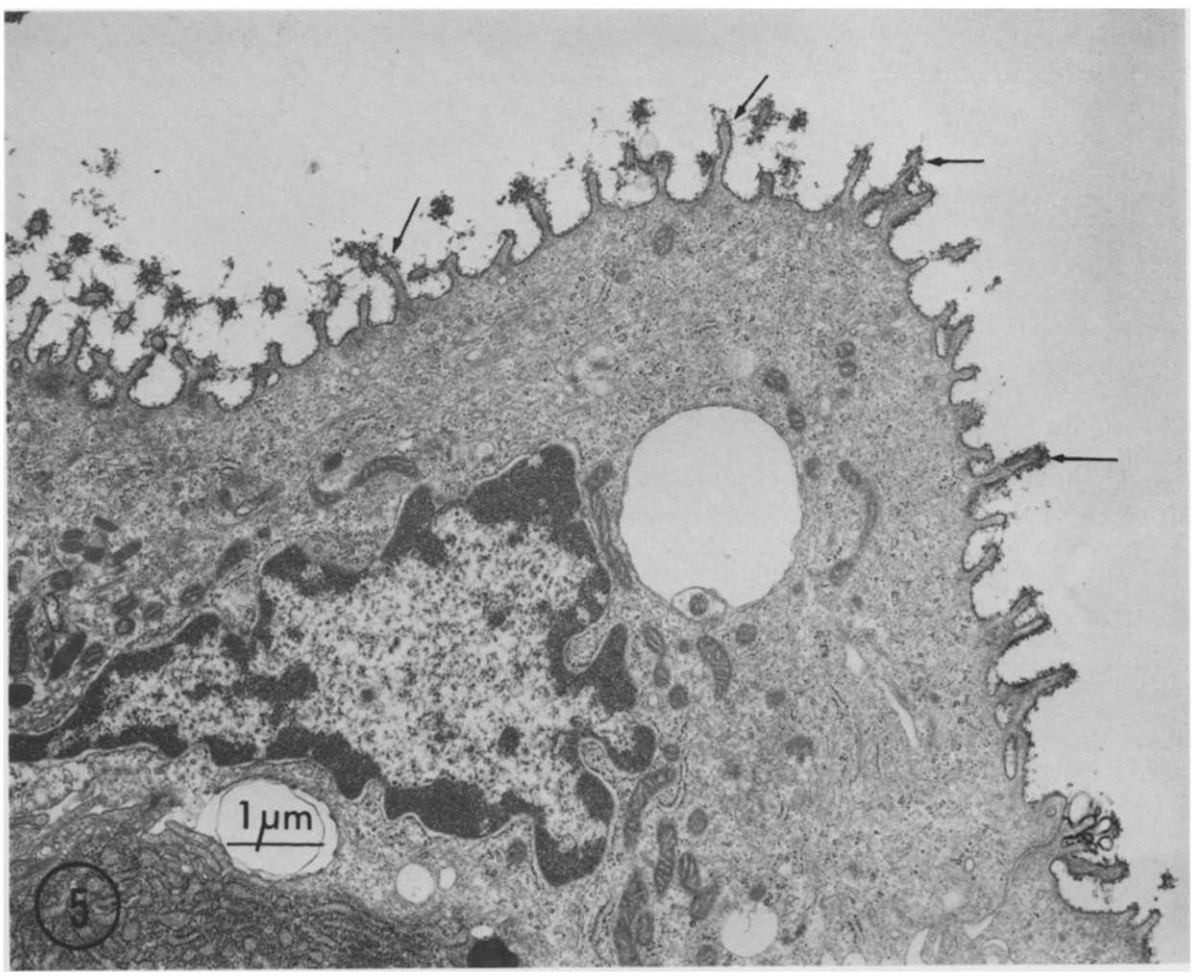


Fig. 5. The variability in granule content at 10 min of PMA (10^{-6} M) is demonstrated by a G cell from a different bladder in which granules are notably absent. The ubiquity of highly elongated microvilli is similar to bladders treated with cytochalasin in the presence of ADH [17] and is much greater than the microvillar rearrangement seen with ADH alone [15,16]. ×12500.

containing bodies (tubules, vesicles and multivesicular bodies) and at 60 min even more peroxidase-containing tubules (and vesicles) are labelled. In three experiments in which the number of endocytic bodies were counted, we found an average of seven endocytic bodies per thin section of cell after 15 min of horseradish peroxidase and PMA, 16 endocytic bodies after 30 min and 37 after 60 min. One or two multivesicular bodies per thin section of cell contain horseradish peroxidase at each of these times. The basal rate of uptake in bladders without PMA is less than 1 peroxidase-containing body/thin section of cell at each time-point. It should be noted that in serial sections of horseradish peroxidase uptake, the majority of the peroxidase-containing 'vesicles' have been identified as cross-sections of tubules [3].

That these structures are endocytic and not membrane invaginations is determined by comparison of the number of bodies containing a fluid phase marker, horseradish peroxidase, taken up from the mucosal solution during the PMA challenge, with the number of bodies stained with Ruthenium red [2]. Ruthenium red is impermeant and therefore defines only membrane continuous with the surface. In PMA-treated bladders fixed with Ruthenium red/glutaraldehyde, Ruthenium red labels few apical structures (two per thin sec-

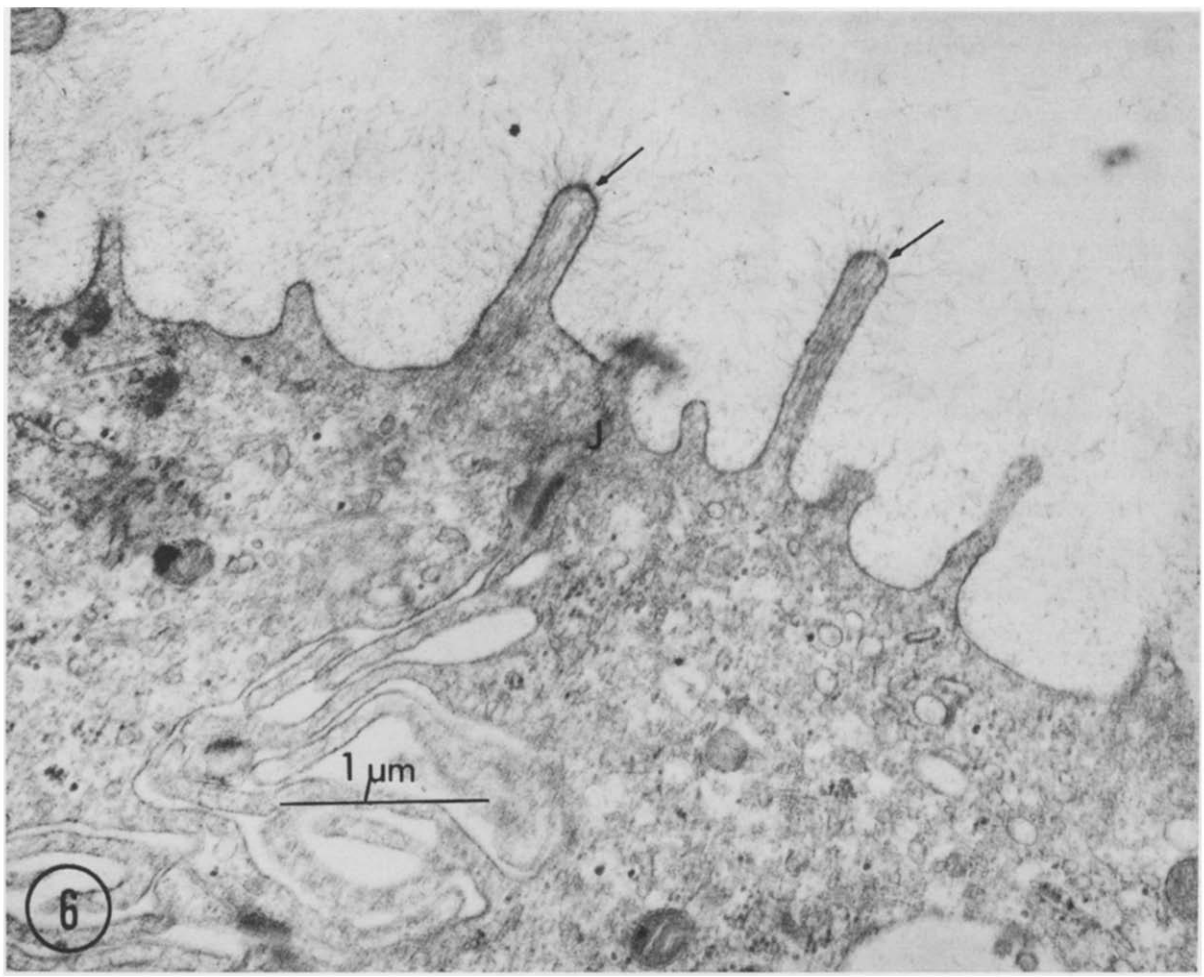


Fig. 6. Apical region of two adjacent cells and their shared tight junction (J) after 10 min of 10^{-7} M PMA in 0.01% DMSO. As contrasted with microvilli on control hemibladders, the microvilli are elongated and have highly oriented actin filaments. The extensive, organized glycocalyx (arrow) on their mucosal surface is typical of PMA-treated bladders. ×30000.

tion of cell) (Fig. 8), although there are many small vesicles in that region. And contrary to peroxidase uptake, the number of Ruthenium red-delineated vesicles does not increase with time of PMA treatment prior to fixation. For example, although on average only two structures have Ruthenium red at 60 min, the average cell has 37 horseradish peroxidase-containing bodies (cf. Figs. 7 and 8). Thus, PMA, like ADH, induces a true endocytosis in the G cells. Some of the large membrane-bounded vacuoles seen after 60 min of PMA contain horseradish peroxidase (Fig. 7). The exclusion of

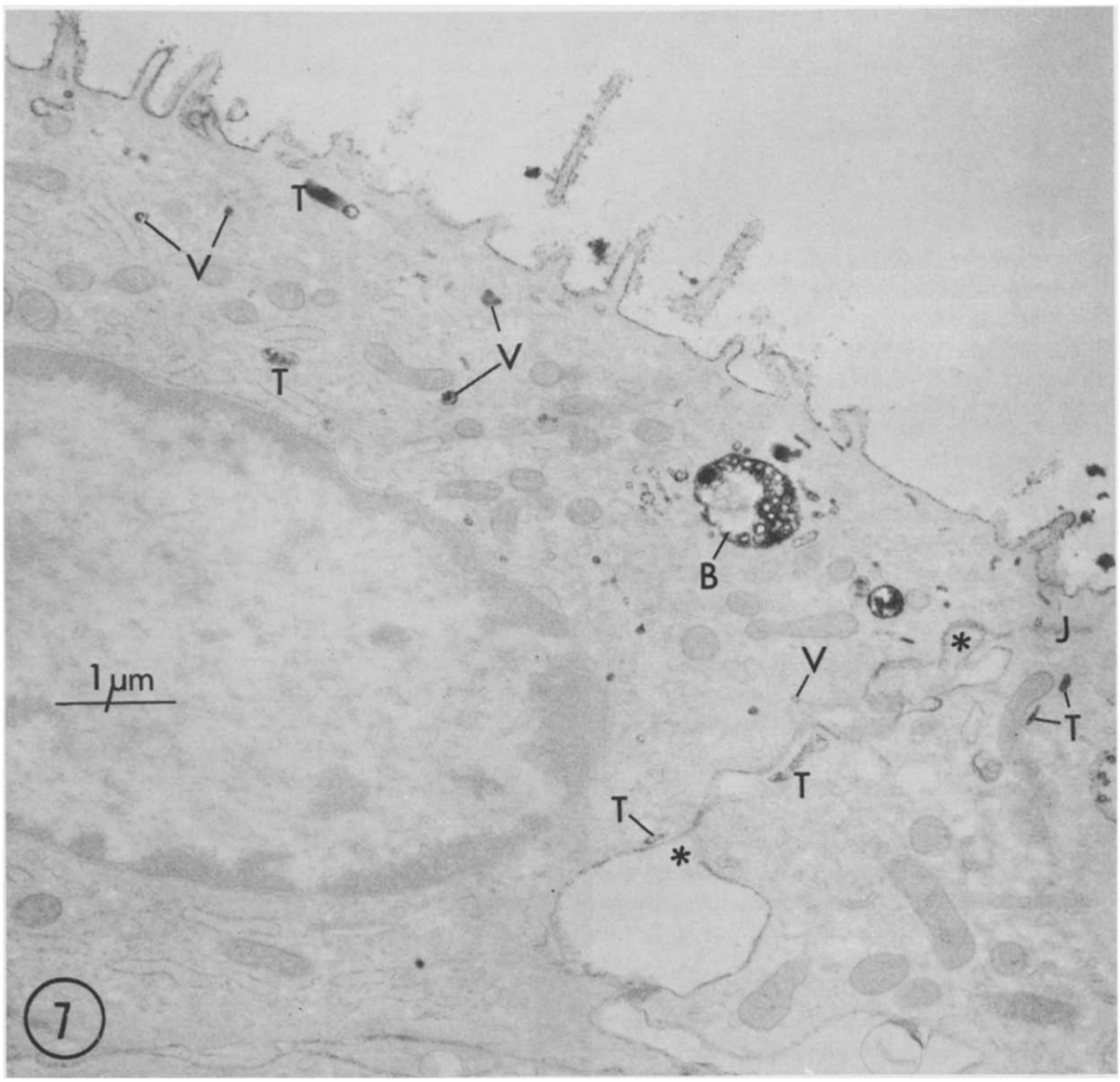


Fig. 7. Horseradish peroxidase reaction production in adjacent granule-rich cells from an hemibladder exposed to 10^{-6} M PMA for 60 min. Horseradish peroxidase is demonstrated in tubules (T) and vesicles (V) as well as multivesicular bodies (B) and is excluded from the tight junction (J). Horseradish peroxidase from the mucosal solution is seen in some intercellular spaces (*) and adjacent tubules and vesicles after mucosal PMA. As seen here and in Fig. 8, the microvilli maintain their highly elongated form even after 60 min of PMA exposure. Control hemibladders (0.1% DMSO for 60 min) do not have horseradish peroxidase uptake bodies. ×22875.

the extracellular marker, Ruthenium red, from these vacuoles (Fig. 8) makes it unlikely that they are contiguous with the intercellular (extracellular) space.

PMA-treated bladders sometimes have horseradish peroxidase in intercellular spaces below the tight junction and in adjacent intracellular tubular structures. This could be the result of transport of the peroxidase via a membrane-bounded transcellular pathway or movement of it directly fom the

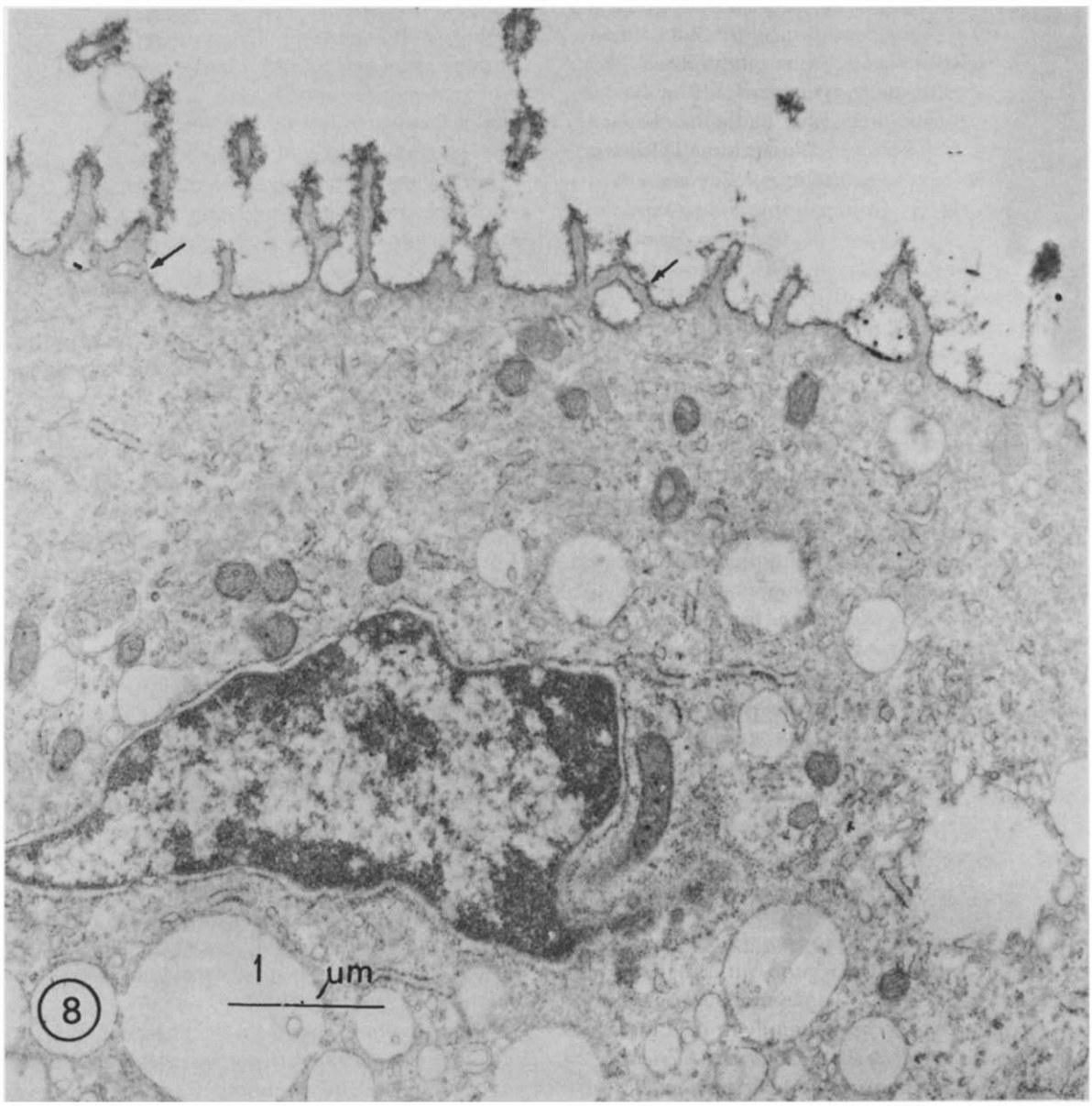


Fig. 8. Granule-rich cell in Ruthenium red/glutaraldehyde/osmium fixed portion of bladder exposed to 10^{-6} M PMA for 60 min. The presence of Ruthenium red (and therefore surface connection at fixation) in some apical vesicles and large cytoplasmic vacuoles is indicated by arrows. Endocytosis, the completed internalization of membrane, is shown by comparison of the many membrane-bounded tubules and multivesicular bodies which contain horseradish peroxidase in Fig. 7 with the relatively few structures containing Ruthenium red after the same experimental manipulation. ×18000.

mucosal solution through the tight junction. The latter possibility is unlikely because the tight junctions exclude both the peroxidase (Fig. 7) and Ruthenium red (not shown). The retention of radioactive mannitol in the mucosal solution of PMA-treated bladders (Hardy, M. and Thayer, A., personal communication) also argues for the junctional tightness.

The mitochondria-rich cell type also responds to mucosal PMA: the lateral dimensions of its apical border, normally a narrow-neck, becomes longer and its microvilli appear to be shorter than the usually elongated form characteristic of this cell type. In contrast to the granular-rich cell type, after PMA treatment peroxidase is seen only in tubules immediately subjacent to the apical surface and is not seen in multivesicular bodies even after 60 min.

Discussion

Mucosal application of phorbol ester stimulates hydroosmosis, exocytosis of the granules in the toad bladder as well as endocytosis, as determined by the uptake of horseradish peroxidase into the granule-rich cells. The degranulation effect of PMA is a greatly exaggerated picture of the usual effect of ADH: whereas a single ADH stimulation releases approx. 40% of the granules [5], PMA releases essentially a cell's entire granule content. Similarly, the elongation of the apical microvilli is greater than that produced by ADH [15–17] and this may be in part an accommodation to the great amount of granule membrane fusing with and presumably incorporated into the apical surface. PMA-induced endocytosis into tubules and multivesicular bodies is comparable in magnitude and timing to the ADH-induced endocytosis [5]. The presence of horseradish peroxidase in the intercellular spaces after PMA exposure is of interest. Mucosal horseradish peroxidase has been seen rarely in intercellular spaces in ADH-treated bladders and then cell damage was detected in adjacent sections [3]. In PMA-treated bladders, although horseradish peroxidase may be seen in the intercellular space this was generally not accompanied by damaged cells containing the peroxidase. It is notable that adjacent to the peroxidase-containing intercellular spaces, are membrane-bounded peroxidase-containing tubules; tubular endocytic structures are characteristic of the apical (ADH-induced) endocytosis [3], while vesicular rather than tubular structures are characteristic of basal-lateral endocytosis. These results could be derived from membrane-associated transport of horseradish peroxidase from the apical to the basal-lateral border induced by PMA. It is of interest that in MDCK cells, longer exposure to PMA disrupts the cytoskeleton, opening the tight junctions and resulting in the flattening of microvilli [18].

Phorbol ester has been shown to stimulate protein kinase C activity implicated in secretion in other cells [19,1,2]. PMA activation of protein kinase C may result in the phosphorylation of cytoskeletal protein(s), e.g., vinculin or actin. Another candidate for phosphorylation is the protein referred to as band 4.1, which interacts with both actin and integral membrane protein. This protein is phosphorylated by protein kinase C [20] and is present in epithelial cells of the toad urinary bladder (Sapirstein, V. and Masur, S.K., unpublished observations). Phosphorylation of such proteins could result in a reorganization [21] of the network in which are embedded the granules and tubular membranes containing intramembranous particle aggregates [22,23] and cause premature exocytic incorporation into the apical membrane perhaps altering their ability to interact positively with other components for functional association. Since we find that PMA also induces endocytosis, it is possible that phosphorylation-dependent cytoskeletal events may be involved in endocytosis as well.

PMA alters the bladders' response to ADH. ADH-induced hydroosmosis after treatment with PMA appears to be more rapid; however, during the first 10 min after ADH application, the rate of increase in hydroosmotic flux is the same in both sets of hemibladders. The maximum is reached earlier in the PMA-treated tissues and occurs at a lower level of stimulation. It is possible that the 'premature' maximum and the inhibition in the ADH-induced increment may be related phenomena. Since ADH can stimulate V1 and V2 receptors, it is clear that the hormone may act through receptor-mediated activation of polyphosphoinositide breakdown leading to protein kinase C activation, as well as adenylate cyclase stimulation and

cAMP-dependent kinase [24]. In our system, direct activation of protein kinase C by PMA may deplete effector units and/or cause their premature insertion before they can be completely activated by cAMP-dependent phosphorylation. In addition, phosphorylation of cytoskeletal proteins in the apical region may pose an impediment to increased hydroosmotic flow which occurs through this region [25]. Alternatively, local pH changes as a result of altered Na^+/H^+ exchange [26], energy depletion, and/or production of an inhibitor of hydroosmosis may be involved. Prostaglandins modulate the ADH response [27], and in other systems, prostaglandin synthesis can be stimulated by PMA-induced activation of arachidonate release [28]. It is possible, therefore, that the inability of PMA to mimic the full ADH response is the result of PMA stimulation of prostaglandin synthesis. However, pretreatment of bladders with a prostaglandin synthesis inhibitor (indomethacin) did not increase the PMA-induced hydroosmosis (Masur, S.K. and Thayer, A., unpublished data).

It is noteworthy that a substantial portion of the toad bladder's response to serosal ADH (exocytosis, endocytosis and an induced hydroosmosis) can be achieved solely by PMA interacting from the mucosal side of the cell. However, the characteristics of PMA-stimulated hydroosmotic response, slower and less than with ADH, indicate that these final events at the apical surface – exocytosis and endocytosis – are not sufficient for the normal, complete hormonal response. Since phorbol esters in other systems neither generate cAMP nor increase in the short term, intracellular Ca [29], the absence of increase of such intracellular messengers may be responsible for the diminished hydroosmotic response. In contrast to PMA, ADH may initiate processing of effector units as well as inducing membrane cycling. PMA, by rapidly initiating exo- and endocytosis, may deplete the cell of the potential effector units in a premature state so that the physiological effect is reduced.

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