Signal Transduction by the Polymeric Immunoglobulin Receptor Suggests a Role in Regulation of Receptor Transcytosis

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Abstract. Many membrane traffic events that were previously thought to be constitutive recently have been found to be regulated by a variety of intracellular signaling pathways. The polymeric immunoglobulin receptor (pIgR) transcytoses dimeric IgA (dIgA) from the basolateral to the apical surface of polarized epithelial cells. Transcytosis is stimulated by binding of dIgA to the pIgR, indicating that the pIgR can transduce a signal to the cytoplasmic machinery responsible for membrane traffic. We report that dIgA binding to the pIgR causes activation of protein kinase C (PKC) and release of inositol 1,4,5-trisphosphate (IP3). The IP3 causes an elevation of intracellular Ca. Artificially activating PKC with phorbol myristate acetate or poisoning the calcium pump with thapsigargin stimulates transcytosis of pIgR, while the intracellular Ca chelator BAPTA-AM inhibits transcytosis. Our data suggest that ligand-induced signaling by the pIgR may regulate membrane traffic via well-known second messenger pathways involving PKC, IP3, and Ca. This may be a model of a general means by which membrane traffic is regulated by receptor–ligand interaction and signaling pathways.

It has been known for decades that certain steps in membrane traffic, such as exocytosis of synaptic vesicles and dense core secretory granules, are highly regulated. However, in recent years it has become apparent that almost every "constitutive" step in membrane traffic is subject to regulation by intracellular signaling mechanisms (for review see Mostov and Cardone, 1995). In many cases this regulation has been uncovered by pharmacological manipulations. For example, treatment with agents that specifically act on heterotrimeric G proteins, such as cholera or pertussis toxins, has been shown to affect traffic through the Golgi (Stow et al., 1991). In some instances genetic methods have been used, e.g., a phosphatidyl inositol 3-kinase (PI3K)[1] has been demonstrated to play a role in delivery of proteins to the yeast vacuole (Schu et al., 1993). However, there is a paucity of data to tie specific receptors and signaling pathways to the physiologic regulation of individual steps in membrane traffic.

As a model system to study regulation of traffic, we have been using the polymeric immunoglobulin receptor (pIgR), which transcytoses dimeric IgA (dIgA) from the basolateral to the apical surface of polarized epithelial cells (Mostov, 1994; Mostov and Cardone, 1995). The cytoplasmic domain of the pIgR contains a basolateral targeting signal, which serves to direct the pIgR to the basolateral surface from either the TGN or the endocytotic pathway (Aroeti et al., 1993; Casanova et al., 1991). Phosphorylation of Ser664 in this signal inactivates the signal and permits the pIgR to be transcytosed (Casanova et al., 1990; Aroeti and Mostov, 1994). Independently of phosphorylation of Ser664, transcytosis is also stimulated by binding of the ligand, dIgA, to the pIgR (Hirt et al., 1993; Song et al., 1994*a*, 1994*b*, 1995). Stimulation of transcytosis by dIgA binding helps to ensure that as the level of available dIgA changes (e.g., due to inflammation or infection) all of the dIgA presented to the epithelial cells is efficiently transcytosed. The discovery of this dIgA-mediated stimulation has altered the previous view that the pIgR is simply a constitutively transcytosing transporter of dIgA. Rather, dIgA binding must somehow cause the pIgR to transduce a signal to the intracellular trafficking machinery responsible for transcytosis.

Transcytosis of dIgA is also regulated by at least two general intracellular signaling mechanisms. First, activa-

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1. *Abbreviations used in this paper*: BAPTA-AM, 1,2-bis(*O*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetraacetoxymethyl ester; Ca_i, intracellular free calcium; DAG, diacylglyceride; dIgA, dimeric IgA; IP3, inositol 1,4,5-triphosphate; PI-PLC, phosphatidyl inositol–specific phospholipase C; PI3K, phosphatidyl inositol 3-kinase; pIgR, polymeric immunoglobulin receptor; PKC, protein kinase C.

tion of the heterotrimeric Gs protein stimulates transcytosis (Bomsel and Mostov, 1992, 1993). At least a portion of this stimulation is due to the production of cAMP and activation of protein kinase A (Hansen and Casanova, 1994), although indirect data suggest that Gsα and Gβγ may play an additional, cAMP-independent role (Bomsel and Mostov, 1993). Second, treatment of MDCK cells with PMA activates the α and ε isozymes of protein kinase C (PKC), and these in turn stimulate transcytosis (Cardone et al., 1994). Both the Gs and PKC pathways seem to stimulate not only pIgR transcytosis, but more generally apical delivery of a variety of molecules in the transcytotic, recycling, and biosynthetic pathways (Cardone et al., 1994; Eker et al., 1994; Hansen and Casanova, 1994; Pimplikar et al., 1994; Pimplikar and Simons, 1994).

What are the signaling pathways involved in the stimulation of pIgR transcytosis by dIgA binding? Given the ability of the PKC and Gs pathways to stimulate transcytosis, it seemed reasonable to hypothesize that dIgA binding to pIgR might lead to activation of one of these pathways, and thereby cause stimulation of transcytosis. We now report that dIgA binding to the pIgR gave a rapid activation of PKCε, production of inositol 1,4,5-trisphosphate (IP3) and elevation of intracellular free Ca (Ca_i). We further show that artificially raising Ca_i with the drug thapsigargin stimulates transcytosis, while chelating intracellular Ca with 1,2-bis(*O*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA-AM) inhibits transcytosis.

Materials and Methods

Cell Growth

MDCK type II cells and MDCK cells that have been stably transfected with the cDNA for rabbit pIgR were maintained on plastic 10-cm dishes, using MEM (UCSF Cell Culture Facility), 5% FBS, penicillin, and streptomycin, as previously described (Cardone et al., 1994). Cells were plated at confluent density onto Transwells (Corning-Costar Corp., Cambridge, MA), 0.4 μm pore size, of various diameters.

Activation of PKC in Response to dIgA Binding to the pIgR

We used a very similar approach to our previous study of the activation of PKC by PMA. Cells were grown on 75-mm Transwell filters for 5 d. Dimeric human myeloma IgA (300 μg/ml, kindly provided by Prof. J.-P. Vaerman, Catholic University of Louvain, Brussels, Belgium) was added to the basolateral medium at 37°C for the times indicated. The zero time control did not receive dIgA. Mouse IgG at 300 μg/ml was used as a control. The filters were then transferred to 4°C and the medium removed. Cells were scraped from the filters in 0.7 ml ice-cold homogenization buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 17 μg/ml PMSF, 5 μg/ml each aprotinin, soybean trypsin inhibitor, and leupeptin) and homogenized. Homogenates were centrifuged at 10^5 *g* for 30 min and the cytosolic supernatant was collected. The pellets were resuspended in 0.7 ml homogenization buffer containing 0.1% Triton X-100. Equal amounts of protein were run on 10% gels, followed by blotting and probing with an isozyme-specific polyclonal antibody to ε PKC (GIBCO BRL, Gaithersburg, MD) and iodinated protein A. The distribution of the isozyme in the soluble and pellet fractions was quantitated using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Production of IP3 in Response to dIgA Binding to the pIgR

MDCK cells expressing pIgR were grown on 24-mm Transwells for 4 d in MEM containing 5% FBS. The cells were then transferred into MEM containing 6 mg/ml BSA in place of the FBS (MEM-BSA) for 24 h. Cells were treated with 10 mM LiCl in MEM-BSA for 10 min before the assay and then their basolateral surface was exposed to dIgA at 300 μg/ml in MEM-BSA and 10 mM LiCl for the indicated times at 37°C. In certain experiments the dIgA was added to the apical medium, instead of the basolateral medium. Filters were removed and tumbled for 5 min in 20% TCA at 4°C. The TCA extract was then centrifuged at 15,000 *g*. The TCA was extracted four times with diethyl ether. The IP3 concentration was determined with a competitive binding assay kit (Dupont-NEN, Boston, MA). Measurements were adjusted to protein (dye binding assay, Bio-Rad Laboratories, Richmond, CA) in the TCA precipitate from each sample. Where indicated, cells were pretreated with 33 μm nocodazole (Sigma Chemical Co., St. Louis, MO) for 1 h at 4°C, or with 50 μg/ml leupeptin (Chemicon International, Inc., Temecula, CA) for 1 h at 37°C.

Effect of Thapsigargin or BAPTA-AM on dIgA Transcytosis

The assay to follow the fate of a cohort of preinternalized dIgA was performed as previously described (Cardone et al., 1994). Briefly, cells grown on 12-mm Transwells were allowed to internalize [^{125}I]dIgA from the basolateral surface for 10 min. This and subsequent steps were at 37°C, using MEM-BSA in Hanks' salts and 25 mM Hepes, pH 7.4. Cells were then washed for 5 min, so that >95% of the [^{125}I]dIgA associated with the cells is intracellular. The release of [^{125}I]dIgA into the apical and basolateral medium over a 2 h time course was then measured. [^{125}I]dIgA released apically is transcytosed dIgA, while [^{125}I]dIgA released into the basal medium is recycled dIgA. Where indicated, 5 μM thapsigargin (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA) was included in the apical and basolateral media during the 2-h chase. Alternatively, cells were pretreated with 50 μM BAPTA-AM (BIOMOL Research Laboratories, Inc.) for 1 h before the internalization of dIgA and the BAPTA-AM was present throughout subsequent steps. The pretreatment is necessary to give time for the BAPTA-AM to permeate into the cells and be cleaved to the active BAPTA. The total amount of ligand endocytosed was not changed by this treatment.

Fluorescence Techniques

Equipment. The basic components of the experimental apparatus consist of the following: an inverted epifluorescence microscope (Diaphot, Nikon Inc. Instrument Group, Melville, NY) equipped with a 75 W Xenon lamp, Nikon CF Fluor ×100/1.3 NA oil immersion objective, fluorescence excitation filter wheel (Metal Tek) coupled to an additional filter wheel (Metal Tek, Morrisville, NC) holding a variety of neutral density filters, computer controllable excitation light shutter, and a cooled CCD camera (Princeton Scientific Instruments Inc., Monmouth Junction, NJ) with a frame transfer chip (EEV-37) and 12-bit readout. In addition, for some experiments a CCD camera (CCD 200; Video Scope International Ltd., Washington, DC) attached to an image intensifier (VS2525, Video Scope International Ltd.) was used. The emitted fluorescence signal is relayed as real time continuous output to an IBM PC/AT compatible clone and the image pairs were collected on a Sierra Pinnacle Micro optical disk drive (1.3 GByte) where data can be stored for future analysis on a pixel-by-pixel basis using the Metafluor software package (Universal Imaging Corp., Chester, PA). Quantitative image pairs at 340 and 380 nm excitation with emission at 510 nm, were obtained every 15 s over a period of ~15 min. Neutral density filters coupled to the excitation filters minimized the transmitted excitation light to between 1 and 10% depending on the experiment, to minimize photobleaching and photodynamic damage to the cells. The individual wavelength intensities were stable over the experimental time course. The fluorescence excitation was shuttered off, except during the brief periods required to record an image. To correct for intrinsic autofluorescence and background, background images were obtained on a new shard of filter (see below) containing a confluent monolayer of cells that was not loaded with the dye, using the exact acquisition configuration used for the experimental protocol. These background images were subtracted from the corresponding images of cell fluorescence and background corrected ratios were generated for data analysis.

Fura-2 Loading and Cell Preparation for Imaging. MDCK cells were grown to confluence (2–3 d after plating) on Nunc aluminum oxide inserts (25 mM diam) and maintained in culture media (DMEM) containing 5% FBS and 2% penicillin-streptomycin. Cells maintained at room temperature in Hepes buffered salt solution (137 mM NaCl, 4 mM KCl, 10 mM Hepes, 11 mM glucose, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4) were loaded with the membrane-permeant form of the intracellular Ca indica-

tor fura-2 acetoxymethyl ester (5 μM) and the organic anion transport inhibitor, probenicid (3 mM) for 30 min at room temperature. The presence of probenicid was necessary to inhibit substantial leakage of dye out of the cells. Others have also observed significant leakage of fura-2 out of cells due to a probenicid inhibitable organic anion transporter (Di Virgilio et al., 1988, 1990; Millard et al., 1989). After loading with the dye the cells were then allowed to sit for an additional 30 min at room temperature in the probenicid containing salt solution before beginning the experimental protocol. Probenicid was present throughout the entire experimental protocol.

A shard of filter containing a confluent monolayer of fura-2–loaded MDCK cells was then transferred apical side down to a flow through superfusion chamber that was fitted on the top and bottom with a 22-mm square standard glass coverslip (No. 1). The chamber was mounted on the stage of the inverted epifluorescence microscope. Hepes buffered salt solutions were gravity fed into a water-jacketed heating coil to bring the superfusate to a temperature of about 37.5°C before entering the entrance port of the chamber. The cells were maintained at a temperature of 37.0°C for the experiments. Cells were first visualized under transmitted light with a Nikon CF Fluor ×100/1.3 NA oil immersion objective before starting the fluorescence measurements. Dimeric IgA or canine IgG (Sigma Chemical Co.) were added to the perfusion reservoir for a final concentration of 0.1 mg/ml.

Calibration of Signal. To correlate a change in the 340/380 ratio with relative changes in the intracellular Ca values (Ca_i) an in situ calibration of the fura-2 signal was performed in some experiments as previously described (Nigam et al., 1992; Silver et al., 1993). Briefly calibration of the fura-2 signal was carried out in the presence of the Ca ionophore ionomycin (10 μM) in the presence of the Hepes buffered salt solution containing either 1.5 mM Ca (experimental superfusate) or 2 mM EGTA titrated to pH 7.4. Intracellular Ca levels were calculated as described by Grynkiewicz et al. (1985). Each cell in the field was calibrated on a pixel-by-pixel basis with its own corresponding R min and R max value according to this technique.

Results are presented as means ± SEM where *n* equals the number of cells. The means represent the average of individual cells. Comparisons were made by a paired *t* test.

Results

dIgA Binding to the pIgR Causes Activation of PKC-ε

We have found previously that treatment of MDCK cells with PMA causes activation of PKC-ε and -α (Cardone et al., 1994). Activation can be measured by the translocation of PKC from the cytosol to particulate anchoring receptors for activated C kinase (RACKs) (Mochly-Rosen, 1995). We found that addition of dIgA to MDCK cells that had been transfected with the pIgR caused a rapid translocation of PKC-ε from the soluble to the particulate fraction (Fig. 1). Translocation peaked at 3.5 min. Whereas PMA causes a permanent activation of PKC and eventually its down-regulation, physiological activation with diacylglyceride (DAG) is generally short-lived, consistent with our observed results. We did not observe any translocation if control IgG was used in place of dIgA.

In some experiments translocation of PKC-α was also observed. However, for unknown reasons this result was quite variable between experiments (unlike the reproducible results with PKC-ε), and so we cannot be sure of the involvement of PKC-α.

dIgA Binding Causes Production of IP3

PKC-ε is classically activated by DAG, and there is preliminary data that PKC-ε is also activated by phosphatidyl inositol 3,4,5-trisphosphate (Kapeller and Cantly, 1994), a product of phosphatidyl inositol 3-kinases (PI3K). DAG is commonly produced by two pathways. One path is hydrolysis of phosphatidyl inositol 4,5-bis-phosphate (PIP2) by phosphatidyl inositol–specific phospholipase C (PI-PLC) to DAG and IP3, as described above. The other is hydrolysis of phosphatidylcholine by phospholipase D to phosphatidic acid, which is then hydrolyzed to DAG. If the PI-PLC pathway is involved, we should also find that IP3 is produced. As shown in Fig. 2, addition of dIgA to the pIgR-expressing MDCK cells resulted in production of IP3. Production peaked at 5 min, and soon declined almost to baseline. As controls for specificity, IgG (not shown) or

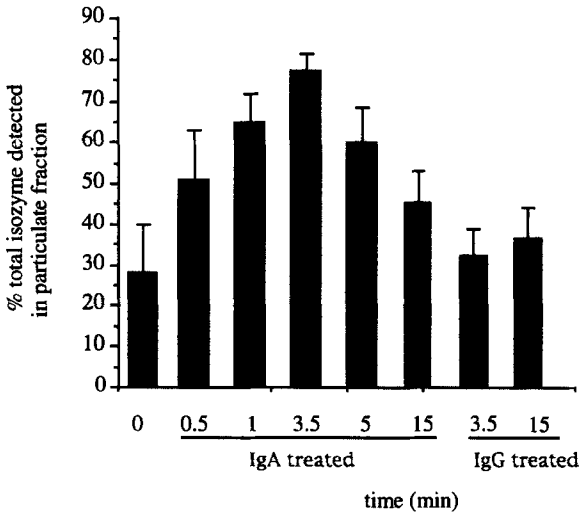


Figure 1. Translocation of PKC-ε from the cytosol to a pellet fraction in response to dIgA binding. MDCK cells were treated with dIgA basolaterally for 0, 0.5, 1, 3.5, 5, or 15 min, or with control IgG for 3.5 or 15 min and then fractionated into cytosol and pellet. Equal amounts of protein from each fraction were subjected to SDS-10% PAGE and blotted using a polyclonal antibodies against PKC-ε. Data are mean ± SE of four separate experiments. Values are normalized so that the total = 100%.

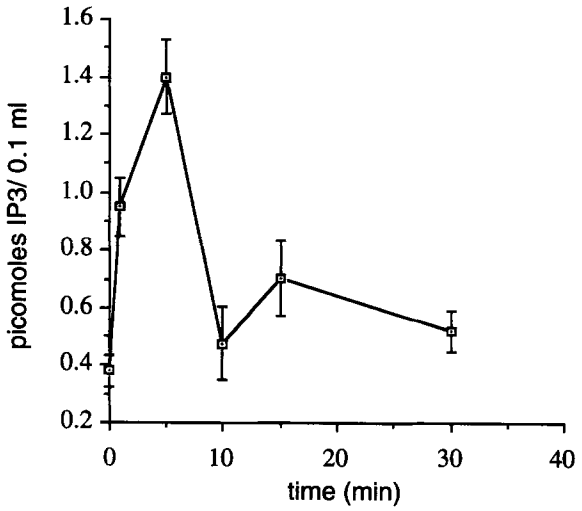


Figure 2. Production of IP3 in response to dIgA binding. Intracellular concentration of IP3 was measured in MDCK cells expressing the pIgR and MDCK control cells exposed basolaterally to dIgA for the indicated times. Data are mean ± SE of quadruplicate filters and are representative of four separate experiments.

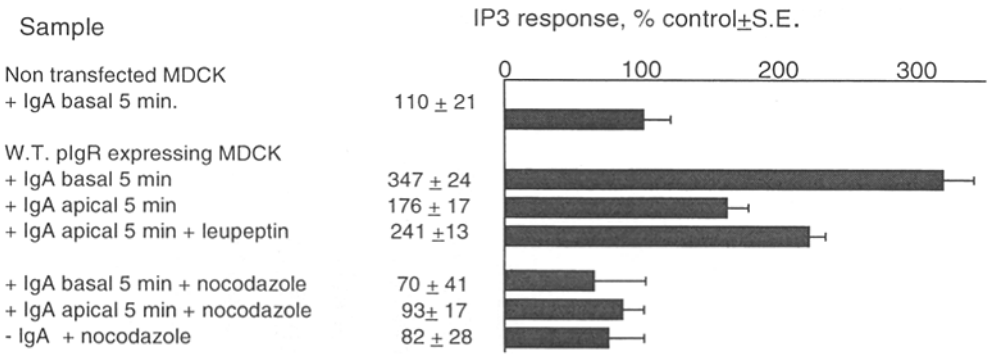


Figure 3. Effects of apical or basolateral addition of dIgA and of nocodazole on IP3 production. IP3 production was measured as described in Materials and Methods. IP3 production is compared with a control value, which was pIgR-expressing MDCK cells not exposed to dIgA. Dimeric IgA (0.3 mg/ml) was added to either the apical or basolateral surface for 5 min. Where indicated, leupeptin or nocodazole pretreatment of the cells was performed, as described in Materials and Methods; these drugs were present throughout the experiment. As an additional negative control, MDCK cells not transfected with the rabbit pIgR were also used. All measurements are the mean of at least five filters.

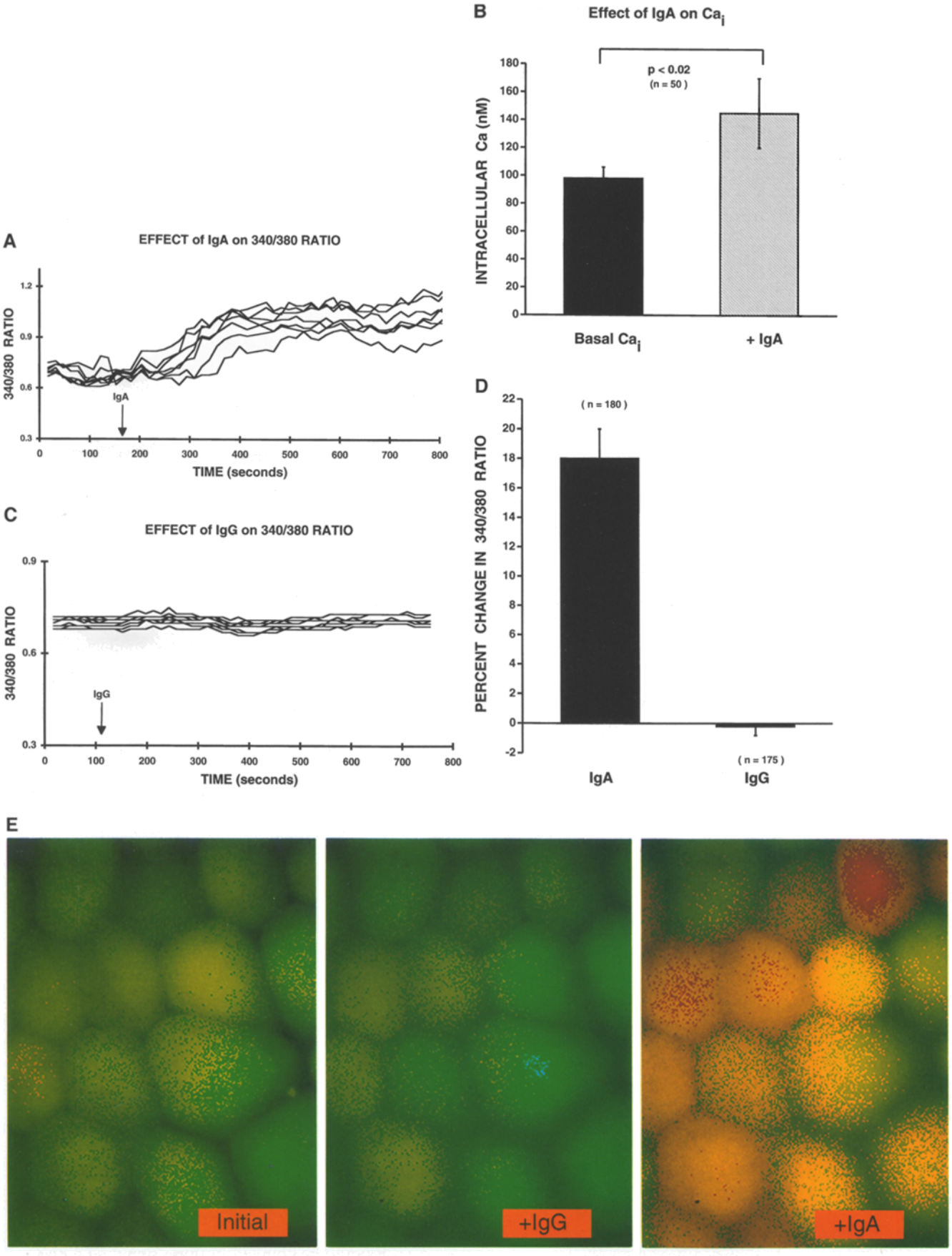
cells not transfected with the pIgR (Fig. 3) gave no response above background.

Although the pIgR is cleaved to secretory component at the apical surface, this cleavage is relatively slow, so there is a pool of uncleaved receptor at the apical surface. We have found previously that ligand can be bound to this apical pIgR and can be internalized into an apical recycling compartment (Breitfeld et al., 1989). Moreover, we reported that the size of this pool can be increased by treatment of the cells with leupeptin, which inhibits the protease that produces secretory component. We found that addition of dIgA to only the apical surface of pIgR expressing MDCK cells also led to the production of IP3 (Fig. 3). Treatment of the cells with leupeptin increased the signal obtained with apically applied pIgR, which is consistent with the accumulation of apical pIgR caused by leupeptin. These data indicate that the pIgR signaling pathway also extends to the pIgR that is at the apical surface or has been endocytosed from that surface. The signal produced by apically applied dIgA was weaker than that resulting from basolaterally applied dIgA, which is in keeping with the smaller amount of pIgR at the apical surface. It seems likely that apical dIgA might also produce a translocation of PKC and an elevation of Ca_i (see below). However, these assays were generally less sensitive and more cumbersome than the IP3 production assay (and in the case of Ca_i measurement, our superfusion apparatus only allowed simultaneous addition of dIgA to both surfaces of the filter-grown cells and did not permit selective addition of dIgA to only the apical surface), so we did not pursue using them to detect the weak signal from apical dIgA.

Intact Microtubules Are Required for pIgR Signaling

Microtubules are required for transcytosis of dIgA and pIgR, and for the organization of the endosomal system in polarized epithelial cells (Breitfeld et al., 1990; Hunziker et al., 1990; Apodaca et al., 1994*b*). Experimentally, transcytosis can be divided into at least three steps (Apodaca et al., 1994*a*, *b*; Song et al., 1994*a*): (*1*) internalization from the basolateral surface into basolateral early endosomes; (*2*) transport from basolateral early endosomes to an apical recycling compartment; (*3*) delivery from the apical recycling compartment to the apical plasma membrane. Step 2 is strongly inhibited by the microtubule depolymerization agent, nocodazole, indicating a requirement for microtubules for this step (Hunziker et al., 1990; Apodaca et al., 1994*b*). Furthermore, the apical recycling compartment is normally clustered around the centriole underneath the center of the apical plasma membrane (Hughson and Hopkins, 1990). Nocodazole treatment causes the apical recycling compartment to disperse throughout the apical cytoplasm (Apodaca et al., 1994*b*). This indicates that

Figure 4. Increase in Ca_i in response to dIgA. (*A*) Effect of dIgA on the 340/380 ratio. The ordinate represents the 340/380 ratio and the *x*-axis is time (s). This figure depicts the 340/380 responses of seven individual cells in a field of view exposed at 170 s to dIgA (0.1 mg/ml). Within 90 s an increase in the 340/380 signal was evident in almost all of the cells. By 400 s all of the cells had an increase in the 340/380 ratio corresponding to an increase in Ca_i. This response was sustained for the remainder of the trace. (*B*) Effect of dIgA on Ca_i. The intracellular 340/380 fura-2 signal was calibrated in situ in a subset of cells ($n = 50$). The initial Ca_i values averaged 97.8 nM ± 8 nM. Addition of dIgA resulted in a significant increase in Ca_i to 145 nM ± 25 nM ($P < 0.02$). (*C*) Effect of control IgG on the 340/380 ratio. The *y*-axis is the 340/380 ratio and the abscissa is time in seconds. Canine IgG (0.1 mg/ml; Sigma Chemical Co.) was added at 115 s as shown by the arrow. Over the time course for this experiment there was no effect on the 340/380 ratio in response to IgG. (*D*) Percent change in the 340/380 ratio with dIgA and IgG. Ratio values were normalized as the percent change from the initial baseline value. Addition of dIgA resulted in an overall increase of 18% ± 2% ($n = 180$) in the ratio signal above the baseline 340/380 value. In contrast there was no change in the initial 340/380 ratio with addition of IgG (−0.2% ± 0.6% $n = 175$). (*E*) Pseudocolor ratio images of MDCK cells grown on inserts and loaded with the intracellular Ca indicator, fura-2 visualized at ×100. The pseudocolor corresponds to the ratio values as follows: blue-green = low ratio = low Ca_i, to orange-red = high ratio = high Ca_i. (*Left*) initial basal ratio values in this field of cells. Addition of IgG to the superfusate (center) did not result in an increase in the 340/380 ratio on a cell-per-cell basis as evidenced by the pseudocolor image, rather it suggests a small decrease in the ratio (decreased Ca_i) by the preponderance of green compared with the left panel. Addition of dIgA (*right*) results in an increase in the ratio (increased Ca_i) as indicated by the switch to orange-red pseudocolor.


A
EFFECT of IgA on 340/380 RATIO
340/380 RATIO
IgA
TIME (seconds)
B
Effect of IgA on Ca$_i$
INTRACELLULAR Ca (nM)
p < 0.02
(n = 50)
Basal Ca$_i$
+ IgA
C
EFFECT of IgG on 340/380 RATIO
340/380 RATIO
IgG
TIME (seconds)
D
PERCENT CHANGE IN 340/380 RATIO
(n = 180)
(n = 175)
IgA
IgG
E
Initial
+IgG
+IgA

the normal functional organization of the apical recycling compartment requires intact microtubules. To examine the importance of microtubules in pIgR signaling, we treated cells with nocodazole and then exposed to dIgA at either the basolateral or apical surface. The production of IP3 in response to the dIgA was assessed. As shown in Fig. 3, nocodazole treatment abrogated signaling by the pIgR. A control of nocodazole treatment without dIgA exposure had no significant effect on the IP3 level in the cell.

dIgA Binding to pIgR Causes an Increase in Ca_i

IP3 is a second messenger that causes the release of Ca from intracellular stores, and thus a rise in Ca_i. We used the Ca-sensitive fluorescent dye, fura-2 to measure changes in Ca_i in response to binding of dIgA. Fig. 4 *A* represents a typical recording of the experimentally determined 340/380 ratio as a function of time. In this particular experiment seven cells were individually tracked. The ordinate is the 340/380 ratio as a function of time. The chemistry of fura-2 binding to free Ca dictates that increases in the 340/380 ratio correspond to increases in Ca_i and decreases in the 340/380 ratio correspond to decreases in Ca_i. In the example shown in Figure 4 *A*, dIgA (0.1 mg/ml) was added at the arrow (~170 s). The mean steady-state ratio value before addition of dIgA in this group of cells averaged 0.68 ± 0.01. Upon addition of dIgA the 340/380 ratio began to increase ~90 s after addition of the ligand and then within another 2 min reached a steady-state value which was greater than that measured before addition of dIgA (0.97 ± 0.03 vs. 0.68 ± 0.01). In all experiments the response to dIgA (or, as a negative control, IgG) was followed for 10 min.

As shown in Fig. 4 *A*, the increase in the 340/380 signal was very similar for these seven cells. For a total of 180 cells studied, 141 responded to addition of dIgA; however, the magnitude of the response and rate of increase of the 340/380 ratio were quite variable. We found that cells which were going to show a dIgA-mediated increase in the 340/380 ratio responded within the first 2 min of adding the ligand to the superfusate and generally reached a peak by 200–400 s, after which the Ca_i was often regulated back toward the pre-dIgA level within 10 min. Therefore for each cell studied the 340/380 ratios were compared 60 s before addition of ligand and 200–400 s after addition of dIgA (IgG).

To be sure that the change in the 340/380 ratio observed with addition of dIgA was due to changes in Ca_i, we performed in situ calibrations at the end of several experiments. As shown in Fig. 4 *B*, for a total of 50 cells, addition of dIgA caused Ca_i to increase significantly from 100 nM ± 8 to 145 nM ± 25 ($P < 0.02$). These data demonstrate that increases in the 340/380 ratio with addition of dIgA correspond to increases in Ca_i.

To be certain that the increases in the 340/380 ratio were due to a dIgA-mediated response, experiments were performed where canine IgG was substituted for dIgA. Fig. 4 *C* is a time course of the 340/380 ratio for 10 cells exposed to IgG. The baseline ratio values averaged 0.70 ± 0.001. Addition of IgG (115 s) did not appear to have any effect on the 340/380 signal. As shown in the figure, the mean ratio calculated 265 s after addition of IgG (0.69 ± 0.005) was no different than the initial pre-IgG ratio value.

The quantitative differential responses to dIgA and IgG are summarized in Fig. 4 *D*. Addition of dIgA resulted in an 18% ± 2% (n = 180) increase in the 340/380 ratio above the initial baseline value, while exposure to IgG had no effect on the 340/380 ratio (−0.2% ± 0.6% n = 175). Finally, Fig. 4 *E* presents an actual image of a group of cells, using pseudocolor to represent Ca_i. The left panel shows the baseline of untreated cells, while the center is of negative control cells treated with IgG. The right panel is of cells treated with dIgA. The shift from blue-green indicating low Ca_i in the left and center panels to yellow-red in the right panel is indicative of an increase in Ca_i in each of the cells in this field. These data strongly support the hypothesis that dIgA-induced transcytosis involves an IP_3-sensitive Ca signaling pathway. However, the prolonged elevation of Ca_i after stimulation with dIgA raises the possibility that an additional mechanism(s) may be involved in elevation of Ca_i.

Role of Ca_i in Transcytosis

We next asked if the increase in Ca_i produced by dIgA binding could stimulate transcytosis. We used thapsigargin, which poisons the pump that normally sequesters Ca into intracellular stores. Short-term treatment with thapsigargin is widely used to raise Ca_i (Suzuki et al., 1991). As shown in Fig. 5 *A*, treatment with thapsigargin accelerated transcytosis of pIgR. The effect was greatest, about twofold, at early time points. Neither the ultimate extent of transcytosis nor the recycling of dIgA to the basolateral medium were significantly affected by thapsigargin. These results with thapsigargin are strikingly similar in magnitude and kinetics to those obtained previously with PMA (Cardone et al., 1994). However, down regulation of the Ca-sensitive isozymes of PKC by chronic exposure to PMA did not alter the response to thapsigargin (not shown), indicating that the effect of thapsigargin is not due to a secondary stimulation of PKC.

We further investigated the possible role of intracellular Ca with the drug BAPTA-AM. This compound is membrane permeable. After reaching the cytoplasm, the acetoxymethyl ester groups are cleaved off by intracellular esterases, releasing free BAPTA, a calcium chelator. As shown in Fig. 5 *B*, BAPTA-AM treatment inhibits transcytosis by ~50%, but has little effect on recycling. This suggests that Ca_i probably plays an essential role(s) in transcytosis of dIgA by pIgR.

Discussion

Our previous finding that dIgA binding to the pIgR-stimulated pIgR transcytosis suggested that the pIgR is capable of ligand-induced signal transduction. Here we show that dIgA binding to the pIgR activates several well-known signaling pathways, including PKC-ε, production of IP3, and elevation of Ca_i. We had shown previously that activation of PKC by phorbol ester can stimulate transcytosis, and now we present data that treatment with thapsigargin can cause a very similar stimulation of transcytosis, while BAPTA-AM inhibits transcytosis.

The pIgR could be coupled to these signaling pathways by several possible mechanisms, although as of yet we have no strong evidence to distinguish among these possi-

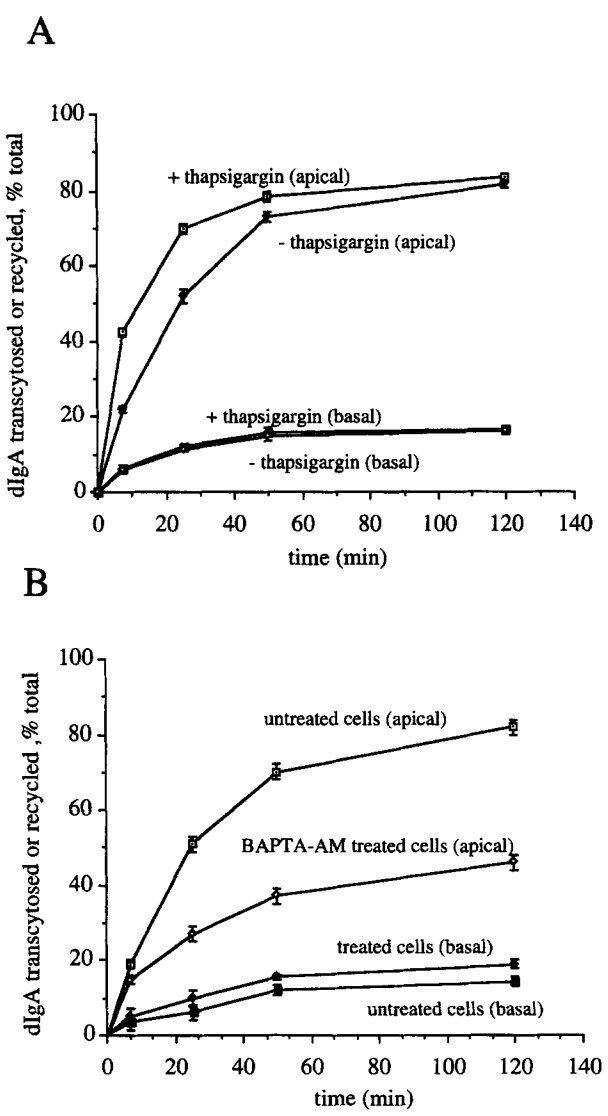


Figure 5. Role of Ca in transcytosis. (*A*) Thapsigargin stimulates transcytosis of dIgA. (*B*) BAPTA-AM inhibits transcytosis. Cells grown on 12-mm Transwells were allowed to internalize [^{125}I] dIgA from the basolateral surface for 10 min at 37°C. Transcytosis and recycling of preinternalized dIgA was then examined over a 2-h chase, as described in Materials and Methods. Where indicated, 5 μM thapsigargin (BIOMOL Research Laboratories Inc.) was included in the apical and basolateral media during the 2-h chase. Alternatively, cells were pretreated with 50 μM BAPTA-AM for 1 h, and BAPTA-AM was included in all subsequent steps. Error bars are SE of triplicate filters.

bilities. The pIgR might activate a PI-PLC, which would hydrolyze PIP2 to DAG and IP3, thereby accounting for our results with PKC, IP3, and Ca_i. There are two well-known mechanisms for activation of PI-PLC. Most single membrane spanning receptors (like the pIgR) activate PI-PLCγ via a pathway involving tyrosine phosphorylation (Cohen et al., 1995). The cytoplasmic domain pIgR does not have any homology to any known tyrosine kinases, and it lacks the "immune receptor tyrosine activation motif" found in many receptors that are coupled to tyrosine kinases (Weiss and Littman, 1994). Furthermore, the pIgR itself is not detectably phosphorylated on Tyr (Hirt et al., 1993; Cardone, M.H., and K.E. Mostov, unpublished data) and has no obvious homologies to consensus recognition sites for SH2, SH3, PTB, or pleckstrin homology domains (Cohen et al., 1995; Kavanaugh and Williams, 1994). It is possible that the pIgR is coupled to a nonreceptor tyrosine kinase(s), either directly or via a second subunit, which in turn recruits the kinase. Many other receptors in the immune system signal in this manner. Another possibility is that the pIgR is coupled to a heterotrimeric G protein, which in turn activates a PI-PLCβ. Although most receptors that activate heterotrimeric G proteins span the membrane seven times, there are examples of receptors that span the membrane once and activate heterotrimeric G proteins (for review see Bomsel and Mostov, 1992).

A nonmutually exclusive possibility is that the pIgR activates a PI3K; this might be via a tyrosine kinase and/or heterotrimeric G protein. The resultant phosphatidyl inositol 3,4,5-phosphate could activate PKC-ε (Kapeller and Cantly, 1994). PI3K has been shown to be involved in a number of membrane traffic events, such as delivery of hydrolases to yeast vacuoles, or the homologous delivery to lysosomes in mammalian cells (Brown et al., 1995; Davidson, 1995). It has recently been shown shown that the PI3K inhibitor, wortmannin, inhibits transcytosis of pIgR (Cardone and Mostov, 1995; Hansen et al., 1995). A caveat is that wortmannin can inhibit other enzymes in addition to PI3K.

When endocytosed from the apical plasma membrane, dIgA and pIgR reach the apical recycling compartment. Here they meet dIgA and pIgR that have been endocytosed from the basolateral surface and are en route to the apical surface. We can imagine that the signaling initiated by the pIgR might take place largely in the apical recycling compartment. Delivery from the apical recycling compartment to the apical plasma membrane (step 3 of transcytosis) is the principal event that is regulated by dIgA binding, PKC, and cAMP (Song et al., 1994*a*; Mostov and Cardone, 1995). Perhaps the pIgR at either cell surface associates with an initial element of the signaling pathway (e.g., a kinase or heterotrimeric G protein) and travels with it to the apical recycling compartment, where downstream signaling events occur. Our experiments with apically bound dIgA and with nocodazole support a role for the apical recycling compartment in signaling. When dIgA is bound to the pIgR at the apical surface and internalized into the apical recycling compartment, pIgR-mediated IP3 response can also occur. Depolymerization of microtubules with nocodazole blocks the delivery of basolaterally added dIgA to the apical recycling compartment, and also causes this compartment to disperse from its normal localization clustered around the centriole underneath the apical plasma membrane (Apodaca et al., 1994*b*). Nocodazole also blocks IP3 signaling by dIgA added at either the apical or basolateral plasma membrane. The relatively slow onset and prolonged duration of the Ca signal are also consistent with signaling taking place in the apical recycling compartment, rather than at the basolateral plasma membrane. Taken together, these data suggest that signaling by the pIgR may be immediately connected with microtubule-dependent delivery of pIgR to the apical recycling compartment, and/or the microtubule-dependent organization of this compartment (Rodriguez-Boulan and Powell, 1992).

The stimulatory effects of thapsigargin on the kinetics and extent of transcytosis are strikingly similar to those of PMA, which was shown to stimulate delivery of dIgA to the apical surface from the apical recycling compartment (Cardone et al., 1994) making it likely that increasing free Ca_i also acts at this step of transcytosis. In pancreatic acinar cells, acetylcholine activates the PI-PLC pathway mobilizing second messenger IP3 and subsequently regionally increasing intracellular Ca_i (Kasai et al., 1993; Thorn et al., 1993; Berridge and Dupont, 1994). Stimulated exocytosis of granules ensues in a manner which coincides spatially and temporally with release of Ca_i (Kasai et al., 1993). The Ca_i signal in MDCK cells may be similarly localized to the apical region of the cell and thereby act on the apical recycling compartment and the last step in transcytosis.

Our results with thapsigargin and BAPTA-AM suggest that Ca plays an important role(s) in transcytosis. The imaging experiments demonstrate that addition of IgA to the superfusate induces an increase in Ca_i in the majority of cells studied. There was considerable heterogeneity in the magnitude and kinetics of this response, which may have been due to a variety of factors such as different stages of cell growth and development that could affect the intracellular Ca stores, and differential amounts of pIgR expressed on the basolateral and apical membranes. Our experimental design did not allow us to differentiate between extracellular influx and intracellular release of Ca that led to the observed increase in Cai. The plasma membrane of the MDCK cells is thought to contain both a Ca-ATPase and a Na/Ca exchanger, but there are no known Ca channels thought to exist there (Bindels et al., 1990). We did not vary extracellular Na at any time point during the experiment, making it doubtful that the activity of the Na/Ca exchanger was altered. The IP3 and thapsigargin data strongly suggest that the source of the increase in Ca_i is from intracellular stores.

Ca_i regulates the exocytosis of synaptic vesicles and dense core secretory granules (Kelly, 1990). Ca_i is also important in a variety of constitutive membrane traffic processes, such as ER to Golgi transport (Beckers and Balch, 1989) and fusion of nuclear envelope vesicles (Sullivan, 1993). Synaptotagmin may be a sensor for Ca that controls fusion events in synapses and other cell types (Li et al., 1995; Sudhof, 1995).

Moreover, cellular events other than transcytosis may be regulated by dIgA binding. For instance, transcription of the pIgR is stimulated by interferon γ, interleukin 4, and tumor necrosis factor a, which indicates that the transport of dIgA is coordinated with other aspects of the immune response (for review see Mostov, 1994). Binding of dIgA might lead to a stimulation of transcription of pIgR, so the production of pIgR could be raised in situations (e.g., inflammation or infection) where the production of dIgA is increased.

The results presented here illustrate how ligand binding to a receptor can regulate membrane traffic through well-known signaling pathways. There are other receptors whose movement is regulated by ligand binding (e.g., EGF, PDGF, and Fc receptors), although unlike the pIgR, the trafficking of these receptors is considered largely secondary to their signaling function (for review see Fantl et al., 1993). We suggest that the pIgR is a model for other membrane traffic processes, which may be regulated by unidentified receptors. These receptors might respond to extracellular ligands, to the lipid composition of the membrane, or to cargo moving through specific compartments. Regulation of traffic by cargo is an attractive homeostatic mechanism, as it enables the cell to adjust to varying amounts of cargo, while maintaining the composition and size of organelles. For example, movement from the ER to the Golgi is highly regulated by several intracellular signaling pathways (Schwaninger et al., 1992). One proposal is that the cargo of newly synthesized (or recycling) secretory or membrane proteins might bind to a receptor in the transitional elements of the ER and trigger the formation of a vesicle that moves to the next station in the secretory pathway. The ligand and receptor involved are so far unknown. Similarly, in delivery from the yeast TGN to the endosome and vacuole, a PI3K is involved (Schu et al., 1993). Perhaps the ligand and receptor there are a newly made vacuolar enzyme and the receptor that carries it to the vacuole. This receptor might somehow activate the PI3K, leading to vesicle formation. We speculate that these systems might be broadly analogous to the one reported here, with the primary differences being that dIgA is provided to the cell externally, rather than being synthesized by the cell, and that we have presented evidence supporting a role for PKC and Ca in stimulating transcytosis.

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