Ca^{2+} mobilization and interlayer signal transfer in the heterocellular bilayered epithelium of the rabbit ciliary body

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1. 'Ratiometric' fura-2 methodology in slice preparations and 'intensitometric' fluo-3 measurements of confocal images were used to simultaneously monitor Ca^{2+} mobilization in the two distinct, apically joined cell layers which constitute the ciliary body epithelium (CBE): the non-pigmented (NPE) and pigmented (PE) epithelia.

2. Both methods yielded comparable results regarding Ca^{2+} responses in the syncytium upon stimulation with adrenergic and cholinergic agonists.

3. The α_1-adrenoceptor agonist phenylephrine elicited a moderate $[Ca^{2+}]_i$ increase in the PE, whereas NPE $[Ca^{2+}]_i$ remained unchanged or exhibited a slight diminution.

4. In combination with carbachol, the α_2-adrenoceptor agonist brimonidine elicited large Ca^{2+} increases (> 10-fold) in both the NPE and PE cell layers, even though previous studies indicated the absence of an α_2-adrenergic effect on $[Ca^{2+}]_i$ in the PE. The onset, as well as the peak of the Ca^{2+} responses in PE cells frequently exhibited a small delay with respect to adjacent NPE cells. No such time difference was observed between adjacent NPE cells.

5. Pre-incubation of the ciliary body in Ca^{2+}-free solution under conditions known to elicit overt NPE–PE separation abolished the α_2-adrenocholinergic response in the PE.

6. Addition of heptanol to the perfusate, to block gap-junctional communication, caused a small $[Ca^{2+}]_i$ decrease in the NPE and a slight increase in PE $[Ca^{2+}]_i$. Subsequently, the Ca^{2+} mobilization in the PE in response to the brimonidine and carbachol combination was either blocked or showed a substantial delay. The Ca^{2+} mobilization in the NPE, in contrast, remained unchanged.

7. We conclude that the heterocellular syncytium exhibits rectificatory behaviour with respect to Ca^{2+} mobilization; responses originating within the NPE are easily transferred to the PE, while the reverse does not occur.

The dual-layered ciliary body epithelium (CBE) is the production site of aqueous humour (AH) which is essential for both maintenance of normal intraocular pressure (IOP) and nourishment of intraocular anterior segment tissues (Bill, 1975; Cole, 1977; Brubaker, 1991). Elevated IOP is the most commonly recognized aetiological factor in the development of glaucoma, and even though it primarily results from impaired outflow, the mainstay of clinical management is the pharmacological downregulation of AH formation (Sugrue, 1989). Thus, for decades, a thorough understanding of the mechanisms that contribute to the formation of aqueous humour and its modulation has been a major concern within the field of ocular physiology. The study of AH formation has been complicated by the anatomical and embryological uniqueness of the epithelium. The two constituent layers, the aqueous-facing non-pigmented (NPE) and the serosal-facing pigmented (PE) epithelia, originate from the invagination of the neural crest that leads to the formation of the optic vesicle. The layers are tightly apposed to each other at their apical membranes. Desmosomal complexes stabilize the contact and gap junctions establish a heterocellular junctional path between them. Homocellular gap junctions within the NPE and PE complete the syncytium (Raviola & Raviola, 1978).

The cellular mechanisms of AH formation are not well understood as yet. Still, it is well established that the two epithelial layers interactively contribute to the process (Wiederholt, Helbig & Korbmacher, 1991; Candia, Shi & Chu, 1991; Edelman, Sachs & Adorante, 1994; Butler, Chen, Stegman & Wolosin, 1994). Such interactions involve the co-operation of transport mechanisms to achieve vectorial transport of ions and/or organic solutes and/or an interplay of intracellular mediators of pharmacological modulation.

Over the last two decades, intracellular Ca^{2+} has been shown to occupy a pivotal role as second messenger (Berridge, 1989). Recent studies of Ca^{2+}-related phenomena have been greatly facilitated by the development of Ca^{2+}-sensitive

optical probes and associated methodologies for non-invasive photometric measurements of free cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) in cells and tissues (Grynkiewicz, Poenie & Tsien, 1985). Using these new tools, Farahbakhsh & Cilluffo (1994) have studied the effects of adrenergic and cholinergic drugs on the $[Ca^{2+}]_i$ of NPE cells in intact ciliary processes. Their main finding was that adrenergic and cholinergic drugs, both of which have minute effects by themselves, together potentiate each other to evoke, in a synergetic fashion, a very pronounced $[Ca^{2+}]_i$ increase. More recently, we reported markedly different pharmacological patterns of Ca^{2+} mobilization in the individual isolated CBE cell layers (Schütte, Diadori, Wang & Wolosin, 1996). Specifically, the isolated PE does not show any sensitivity to α_2-adrenoceptor (AR) agonists; however, it responds well to phenylephrine, an α_1-AR agonist. In contrast, the isolated NPE is sensitive to brimonidine (UK 14304), an α_2-AR agonist, but does not respond to phenylephrine. Additionally, while both isolated cells exhibit modest Ca^{2+} mobilization in response to cholinergic stimulation, the isolated NPE responds to simultaneous exposure to adrenergic and cholinergic drugs with the same dramatic increase of intracellular Ca^{2+} seen in the NPE of the intact tissue. In the PE no synergism between the AR agonists and the cholinergic path is observed.

The experiments performed so far indicate that: (a) the synergetic response is intrinsic to the NPE cell; and (b) the α_1-AR response observed in the isolated PE is not seen in the NPE of the intact CBE. The latter suggests that either the junctions between the two layers do not communicate Ca^{2+} mobilization from one cell to the other or, less likely, that the PE responsiveness to phenylephrine occurred as a consequence of the layer separation process. Until now, this question could not be addressed owing to the optical limitations imposed by the CBE anatomy which, in intact tissue, allow Ca^{2+} measurements only in the superficial NPE cell layer. Furthermore, the methods employed so far were not able to establish the excitatory status of the inner PE during the large, synergetic Ca^{2+} response in the NPE. The present study describes the development of two novel methods to simultaneously record Ca^{2+} events within both layers in an attempt to further our understanding of the physiological interactions between the two distinct layers of the CBE *in situ*. The main focus of this report is on the integration of Ca^{2+} responses and the role of the heterocellular junctional path in this regulatory mechanism.

METHODS

'Ratiometric' Ca^{2+} measurements using fura-2

Albino rabbits were killed by CO_2 asphyxiation, the eyes were enucleated immediately, and the ciliary body dissected free of other ocular tissues as described elsewhere (Wolosin, Chen, Gordon, Stegman & Butler, 1993). The ciliary body was cut into several strips (1·5–2 cm long) and incubated in Ringer solution (mM): 110 NaCl, 3·5 KCl, 1·8 $CaCl_2$, 1·4 $MgSO_4$, 1·5 H_3PO_4, 10 Glucose, 14 sodium gluconate and 38 hemi-Na-Hepes containing 1 μM fura-2 (Grynkiewicz *et al.* 1985) in its acetoxy-methoxylated (AM) form (Molecular Probes) for 30–60 min at room temperature (22 °C). The bulk of the ciliary muscle was carefully dissected away and the strips of ciliary body further cut into smaller pieces (4–5 mm). These pieces were then placed, stroma side up, under the dissecting microscope and a small piece of Millipore filter was gently placed on top of them, which resulted in attachment of the tissue to the filter. The filter was then turned over and trimmed to a size approximately 10 mm × 10 mm, which was cut with a razor blade into ~200 μm thick slices. Four to six such filter-mounted ciliary body sections were placed over a circular 18 mm coverslip containing two parallel tracks of silicone grease to hold the sections in place, and the coverslips were mounted onto a perfusion chamber placed atop the heated stage of an Olympus IMT-2 microscope. The microscope was equipped with high numerical aperture (n.a.) objectives (Zeiss ×25 Multi-Immersion, 0·85 n.a.; Zeiss ×63 Oil, 1·3 n.a.) and epifluorescent, alternating dual-wavelength (340 and 380 nm) illumination for fura-2 measurement.

Fluorescence images derived from each excitation were captured using a high resolution video camera (Hamamatsu, Tokyo, Japan) and processed with an image analysis system (Image 1/fl; Universal Imaging, Philadelphia, PA, USA). During experiments, images resulting from the exposure to excitations at 340 and 380 nm (I_{340} and I_{380}, respectively) and the image of the pixel-by-pixel ratio (R) between both intensities (I_{340}/I_{380}) were displayed on-line (e.g. Fig. 1). Within the entire field displayed on the monitor, areas allowing clear identification of individual NPE and PE cells were selected for on-line quantification of ratio changes.

Solutions were delivered through a multi-channel, gravity-driven superfusion system consisting of 60 ml syringes connected onto an 8-port narrow-bore Hamilton manifold. The syringes contained 10 μM carbachol, 1 μM adrenaline, 3 μM brimonidine (UK 14304, RBI, Natick, MA, USA), 30 μM phenylephrine, heptanol (2·3 or 3·5 mM) or a combination thereof, all made up in standard Ringer solution. Perfusion occurred at a flow rate of ~1–2 ml min^{-1}. Surplus fluid was removed through a suction tube connected to a vacuum. Unless stated otherwise, all chemicals used were obtained from Sigma. Equilibration of intracellular and extracellular calcium for calibration purposes (Grynkiewicz *et al.* 1985) was accomplished at the end of each experiment by introducing 10 mM $CaCl_2$ or 10 mM EGTA, respectively, complemented with 4 μM digitonin and 2 μM thapsigargin (Schütte *et al.* 1996). After obtaining calibration values, Ringer solution containing 0·02% Triton X-100 was used to permeabilize the tissue membranes and wash out all cytosolic fura-2. The remaining tissue auto-fluorescence was used to establish background values.

At the end of each experiment, image files were transferred onto optical discs for permanent storage. 'Pair of intensity' log files for selected areas were acquired later in playback mode, and further imported into a spreadsheet program for background subtraction, calculation of area ratios and conversion of ratios to actual $[Ca^{2+}]_i$ (Schütte *et al.* 1996). Final composite graphs of processed data were assembled in CorelDraw®.

'Intensitometric' Ca^{2+} measurements using fluo-3

Albino rabbit ciliary bodies were dissected free of other ocular tissues and vitreous humour and incubated for 30–60 min in Ringer solution containing 1 mM fluo-3 (Minta, Kao & Tsien 1989; Molecular Probes). Small segments (4–5 mm) were attached onto a Millipore filter which was then trimmed to the approximate size of the deposited tissue segments. The filter section was then affixed onto a coverslip using silicone grease and fitted into a perfusion

chamber. The chamber was then mounted onto the stage of a confocal inverted microscope (Leica CLSM, Wetzlar, Germany) so that the coverslip with the attached tissue occupied the top opening while the bottom was sealed with another coverslip through which the tissue was observed. The drug delivery set-up consisted of a three-channel, gravity-driven system. A long working distance (×40, 0·65 n.a.) water immersion objective (Olympus) was used to obtain the widest range of focal planes possible and thus to facilitate the selection of processes suitable for Ca^{2+} imaging. Measurements were made at single focal planes deep within individual processes as shown schematically in Fig. 2*A*, resulting in a bilaterally symmetrical appearance of the processes with the dual-layered epithelium flanking the endothelium of the central vessel on each side (e.g. Figs 2*B* and 5). Selected fields were scanned ($\lambda_{excitation} = 488$ nm; $\lambda_{emission} > 530$ nm) into a 512 × 512 bit matrix for a clear confocal view of both epithelial layers. For better resolution, actual experiments were run at a ×2 or ×4 line accumulation oversampling mode. Under these conditions, scanning time per line was ~10 (2 × 5 ms) or 20 ms (4 × 5 ms), resulting in a total scanning time of 5 or 10 s, respectively, for each selected area. Since the average diameter of each cell amounted to about 1/10 to 1/20 of the width (or length) of the field, the data collection over each single cell required between 0·25 and 1 s, depending on oversampling rate and the specific dimensions of each cell. The average length of experiments was 5–7 min, corresponding to 60–84 or 30–42 picture matrices in ×2 and ×4 oversampling mode, respectively. Obtained image files were stored in chronological order in TIFF format, imported into Photoshop (Adobe, Mountain View, CA, USA) and analysed, using the mean pixel value of a selected area (defined over an identifiable cell) for intensity changes by means of the pixel histogram subroutine. Numerical data were processed in a spreadsheet and normalized to a starting value of 1 by dividing each entire data column by the first numerical value of emission intensity. For one set of experiments, the ciliary body was incubated for 2·5 h in Ca^{2+}-free Ringer solution before dye loading. This exposure results in the overt, irreversible physical separation of the two layers (Fain, Cilluffo, Fain & Lee, 1988; Wolosin *et al.* 1993; Schütte *et al.* 1996).

RESULTS

Technical considerations

In the intact CBE, the inner location of the PE prevents its *en face* optical measurement of Ca^{2+}. To circumvent this anatomical handicap, we developed two alternative methods. The first approach consists of a slice preparation, whereby the ciliary body is cut orthogonal to the main axis of the processes. The sectioning preserves all anatomical features of the tissue (Fig. 1*A*) and allows both optical and physical access to all components, thus enabling quantitative measurement of Ca^{2+} mobilization in individual cells by fura-2 fluorimetry. The second method employs optical sections of the tissue and allows qualitative measurement of Ca^{2+} events through changes in emission intensity of the single-excitation dye fluo-3.

Both methods have distinct advantages and disadvantages. By screening out effects related to net intensity changes caused by bleaching or leak of the dye, the ratiometric approach allows reliable conversions of intensity signals into $[Ca^{2+}]_i$. On the other hand, using conventional epi-fluorescent illumination, it is not possible to completely rule out contributions of stray light to the intensities measured. In our particular arrangement, where stacks of NPE and PE cells lay above the selected plane of observation, out of focus fluorescence from the NPE could conceivably contribute to the intensities collected from the in focus PE cells, and vice versa. Also, the viability of the outermost (at the surface of the section) epithelial cells in a slice preparation could potentially be impaired by mechanical strains generated during the cutting process. In some experiments mercury lamp flicker introduced small oscillations of the ratiometric traces (e.g. in Figs 4*A* and 7*A*).

The confocal microscope method ensures the absence of out-of-focus contributions to the measured intensities. Additionally, the non-invasive tissue treatment reduces potential dangers of mechanically induced cell damage. On the other hand, the available set-up permitted measurements only with the argon laser-compatible single-excitation dye fluo-3. The resulting intensitometric recordings are not amenable to reliable calibrations; changes in intensity could equally reflect changes in $[Ca^{2+}]_i$ or dye bleaching and/or leaking. The method also sacrifices temporal resolution (1 data point $(5–10\ s)^{-1}$). Furthermore, we observed that in the wake of the synergetic Ca^{2+} responses which bring $[Ca^{2+}]_i$ to the micromolar range, decay of fluorescence was often exacerbated which introduced an additional hindrance to reliable conversions of intensity changes into $[Ca^{2+}]_i$. For these reasons, we decided against converting intensity values into $[Ca^{2+}]_i$ so normalized intensity data are shown instead. Nevertheless, the following observations provided a framework to evaluate the relationship between $[Ca^{2+}]_i$ and the increases in intensity. (a) The introduction of 10 mM Ca^{2+} along with 4 μM digitonin and 2 μM thapsigargin, conditions which saturate the fura-2 response (Schütte *et al.* 1996), increased the fluo-3 intensity by about 3·5-fold (not shown). (b) During synergetic adrenocholinergic responses, in which $[Ca^{2+}]_i$ regularly reaches values between 1 and 2 μM, a ~2·5-fold increase in fluo-3 emission intensity was seen.

Previous studies of rabbit NPE function were performed using pigmented strains. In the present study, however, the large mismatch in fluorescence between the NPE and the pigmented PE cells turned out to constitute a severe obstacle for simultaneous measurements by the image analysis system employed. Due to the limited dynamic range of the 8-bit, 256-grey-level image analysis system used, the PE had no measurable intensity when gain and offset were set to have the NPE fluorescence within the correct sensitive range. Conversely, at the gain and offset levels at which a signal from the PE was measurable, the NPE signal was saturated. Therefore, we switched to albino rabbits as the experimental species. Interestingly, even in this case, despite the obvious lack of pigmentation, the PE, which was easily discernible from the NPE (Fig. 1*A*), showed substantially lower fluorescence (Figs 1*B* and *C*). The same fluorescence intensity difference was apparent in fluo-3-loaded tissues (Fig. 2*B*).

This difference is likely to be caused by the abundance of pigment-free (translucent) granules present in the albino rabbit PE (Tormey, 1963), which, as in the pigmented congener, occupy a large fraction of the cellular space.

Finally, it should be mentioned that the endothelial cells lining the lumen of the vessel showed a high degree of regional variability in their loading intensity (see Fig. 5), suggesting that intraprocessal dye uptake may be highly

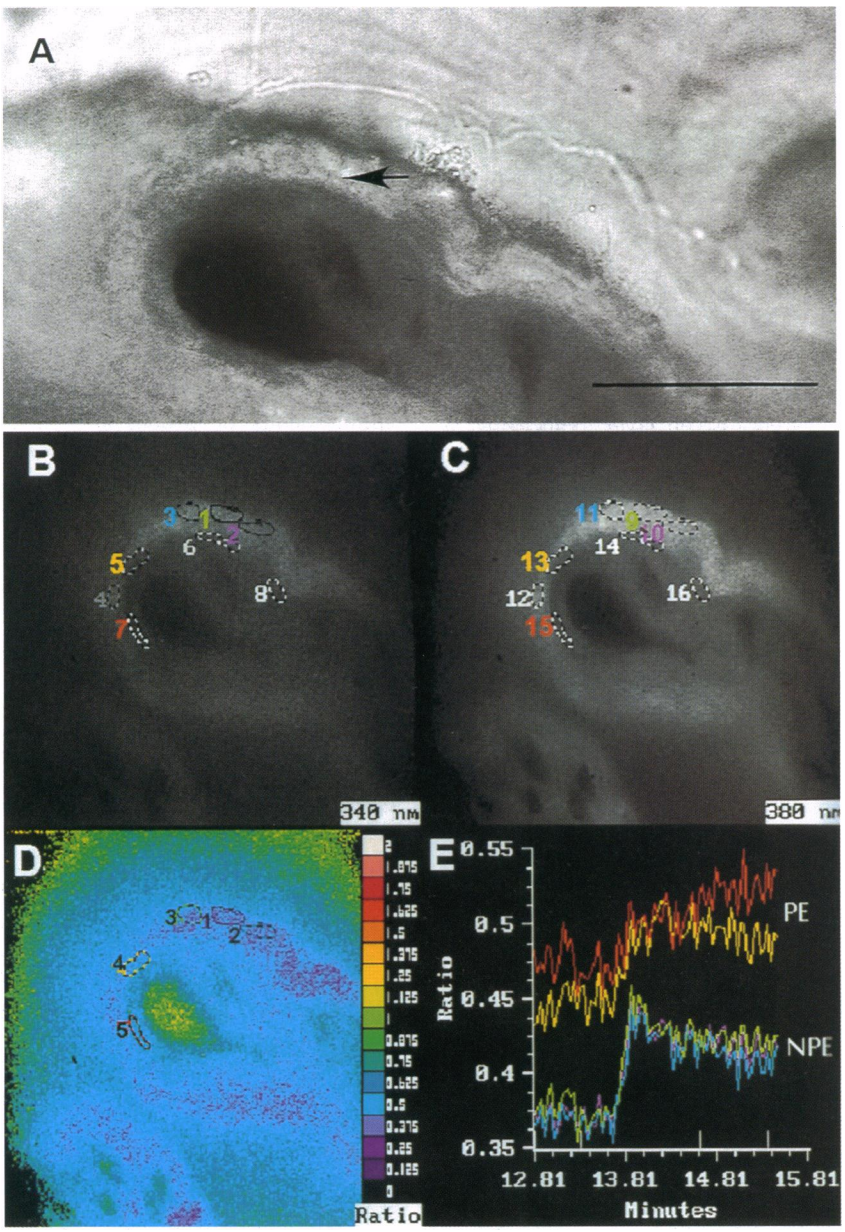

Figure 1. Details of fura-2 ratiometric image analysis of intracellular [Ca^{2+}] in slice preparations

A, transmitted light micrograph of an albino rabbit ciliary process as it appears in a slice preparation. Both layers of the epithelium can easily be distinguished from each other by the size and shape of their cells. The arrow points to the NPE–PE boundary. Note the stromal area and the lumen of the intraprocessal capillary. Scale bar represents 100 μm. *B* and *C*, fluorescent images of the same piece of albino rabbit tissue, captured at alternating excitation with 340 or 380 nm light, respectively. Emission was monitored using a video camera attached to a quantitative image analysis system which displayed ratio images (*D*) and traces (*E*) in addition to the captured fluorescence intensity images. Numbers are coloured to correspond with ratio image in *D* and traces in *E*. *D*, resulting pseudocolour ratio image. *E*, ratio traces of selected cells responding to a mixture of UK 14304 and carbachol. For simplification, the ratio traces have been reduced from 8 to 5 cells (1, 2, 3, 5 and 7 in the 340 nm frame (*B*) which correspond to 9, 10, 11, 13 and 15 in the 380 nm frame (*C*)). Note that the 3 NPE traces (blue, green and purple) can hardly be distinguished from each other, owing to their high degree of synchronization. In contrast, the two PE traces (red and yellow) are slightly asynchronous.

dependent on local tissue microanatomy (e.g. the thickness of the stromal layer between the PE and the wall of the vessel).

Ca^{2+} responses

The α_1-AR agonist phenylephrine induces a substantial Ca^{2+} mobilization in cells of the isolated PE (Schütte *et al.* 1996). In intact ciliary processes, using *en face* photometric fura-2 measurements, no excitatory effect of phenylephrine on the NPE was detected. Therefore, one area of our interest focused on the PE responses in the intact, non-separated tissue. Figure 3*A* and *B* shows examples of simultaneous responses from adjacent NPE and PE cells in the slice preparation, without and with conversion to $[Ca^{2+}]_i$ values, respectively. The PE traces reveal substantial Ca^{2+} mobilization. For selected cells in four such experiments, the $[Ca^{2+}]_i$ in the PE increased from 130 ± 70 to 380 ± 90 nM at peak. These values are almost identical to those measured by photometry in the isolated PE cell layer (Schütte *et al.* 1996). It is also noteworthy that, as observed previously in the isolated PE (Schütte *et al.* 1996), the α_1-AR stimulation increased the amplitude of random $[Ca^{2+}]_i$ fluctuations above baseline, causing an increase in apparent 'noise' of the traces. Comparable results were obtained by optical sectioning using confocal microscopy. In all experiments ($n = 15$), upon stimulation with phenylephrine, we found a consistent increase of the PE fluo-3 emission to $140 \pm 15\%$ of the starting baseline value.

The NPE cells adjacent to the responding PE cells showed minimal changes in their $[Ca^{2+}]_i$. In some cases (e.g. Fig. 3*A* and *C*), the trace indicated an initial small $[Ca^{2+}]_i$ increase (less than 50 nM) in unison with that seen in the PE. In other cases, the NPE response went in the opposite direction of the PE response, that is, while the PE reacted with the normal increase in $[Ca^{2+}]_i$, the NPE displayed an actual reduction of $[Ca^{2+}]_i$. However, the techniques employed in this study, in particular the confocal approach, are limited in their ability to detect small $[Ca^{2+}]_i$ changes. Therefore we expanded our line of experiment using the *en face* photometric approach (Schütte *et al.* 1996). In a total of thirty specimens, 40% of the samples displayed increases (25–50 nM), 33% showed decreases (25–50 nM) and the remaining 27% were essentially unaffected (< 25 nM change in $[Ca^{2+}]_i$). Thus, it can be concluded that, statistically, phenylephrine does not change the NPE $[Ca^{2+}]_i$.

Our second area of interest concerned the relation between Ca^{2+} responses in the two distinct epithelial layers caused by simultaneous or sequential exposure to adrenaline and carbachol. In the slice preparation, the NPE reacted with a dramatic Ca^{2+} mobilization (Fig. 4*A*), similar to that seen in the intact CBE (Farahbakhsh & Cilluffo, 1994; Schütte *et al.* 1996). Notwithstanding the uncertainties of accurate $[Ca^{2+}]_i$ estimations in the non-linear upper end of the sigmoidal conversion function (Grynkiewicz *et al.* 1985), the synergetic response brought the $[Ca^{2+}]_i$ of the NPE cells from $\sim 93 \pm 18$ to 1480 ± 450 nM ($n = 12$). Using the slice, however, it was possible to observe that a similar, simultaneous Ca^{2+} mobilization occurred in the PE. The PE $[Ca^{2+}]_i$ rose in all cases to peak values of at least 10% above the NPE peak $[Ca^{2+}]_i$ ($n = 12$). Upon substitution of UK 14304 for

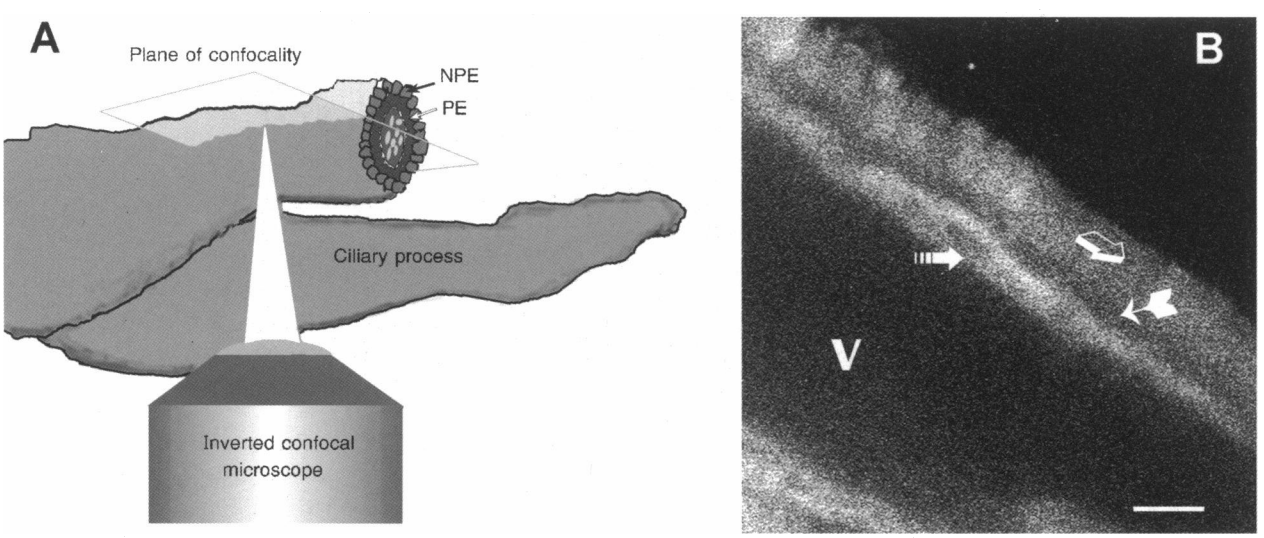

Figure 2. Details of the protocol used for the fluo-3 intensitometric confocal measurements

A, schematic cartoon. Small pieces of ciliary body were scanned on an inverted confocal microscope. The Z-plane of confocality was selected at the centre of individual processes to give a clear view of the flanking bilayered epithelium. *B*, confocal image obtained by ×4 oversampling in line accumulation mode. The palisade-like NPE cells (⇦) stand on the PE layer (➽) and both layers can easily be discriminated due to their different degree of fluo-3 fluorescence. In this particular preparation, the endothelial cells forming the capillary wall (➽) also displayed a high intensity fluo-3 fluorescence. The lumen of the vessel (V) appears empty because red blood cells do not accumulate Ca^{2+} indicator dyes. A small section of the epithelium on the contralateral side is visible at the bottom left corner of the micrograph. Scale bar represents 20 μm.

adrenaline, the responses of both layers showed similar amplitudes and kinetics (Fig. 4*B*, see also ratio traces in Fig. 1). The relevant experiments were carried out in two different ways: either the agonists were introduced for a very short time (~20 s) or they were maintained for up to 3 min. In the first case the mobilization consisted of a single Ca^{2+} spike, peaking within 10–20 s and then receding to control values within 1 min ($n = 5$). In the second case, two alternative response traces could be catalogued. In most cases ($n = 10$), a single large spike was followed by a gradual decrease within 2 min to a near control level. However, in a few cases ($n = 3$), a second Ca^{2+} rise succeeded the first, thereby prolonging the period of elevated $[Ca^{2+}]$ levels, independent of whether adrenaline or UK 14304 was used as the adrenergic stimulus.

Generally, the NPE and PE responses started in unison. Sometimes, a minor delay (1–2 s) between the onset of the NPE and the PE responses was noted. However, because such a delay is within the limit of temporal resolution of our acquisition system, it could not always be confirmed. On the other hand, the peak of the PE Ca^{2+} responses consistently lagged behind the NPE peak (7–12 s). In addition to the temporal differences between the responses of the two layers, there were also some differences in the degree of synchronization of individual cells within each layer (Fig. 1*E*). NPE cells are synchronized to such a point that it becomes impossible to identify the individual traces. The responses in PE cells, however, even though occurring within the same time frame, exhibited a pronounced degree of asynchronism. In the presence of EGTA (10 mM) in the perfusate, Ca^{2+} responses still occurred (Fig. 4*C*). The main difference of these responses compared with experiments in the presence of Ca^{2+} was their very transient nature, i.e. after reaching the peak value, $[Ca^{2+}]_i$ decreased extremely fast and dropped to sub-baseline levels, independent of the stimulus duration.

Within the temporal resolution of the technique, the confocal approach confirmed the basic phenomena observed by the ratiometric method. For example, α_2-adrenocholinergic stimuli elicited Ca^{2+} mobilizations of comparable magnitude within both cell layers, the onset of the mobilizations was essentially simultaneous and the PE peak response trailed

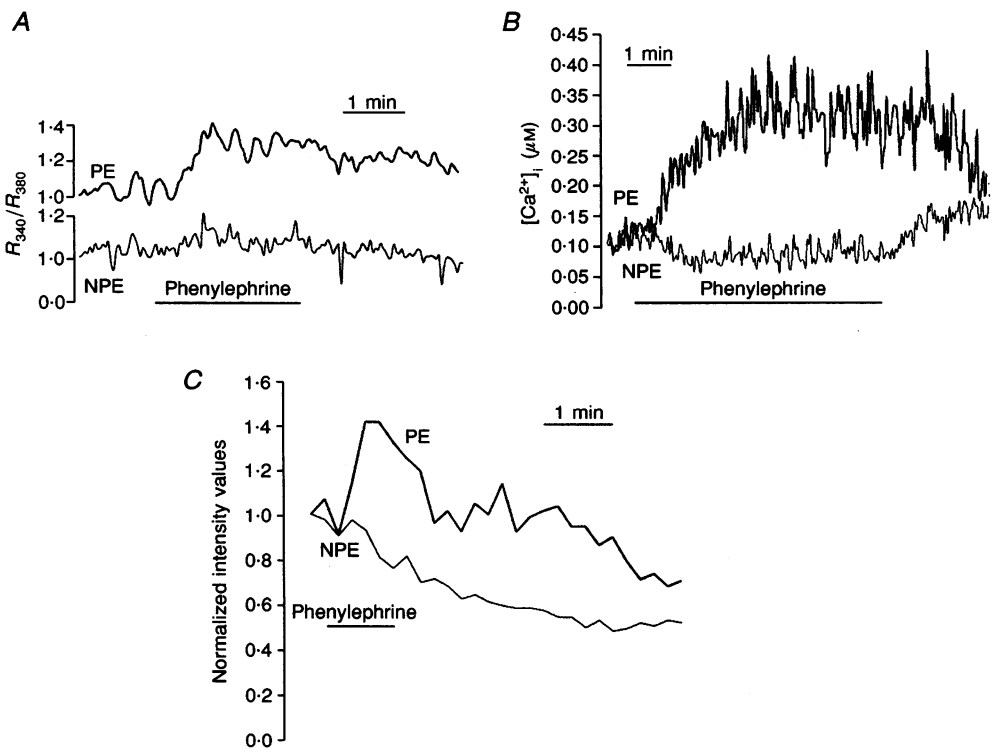

Figure 3. Effect of phenylephrine in both layers of the CBE

A, fura-2-ratio traces of representative PE and NPE responses in a slice preparation upon exposure to phenylephrine; both traces have been normalized to a starting value of 1. The PE shows an increase in *R*. The ratio change in the PE corresponds to a ~3-fold increase in $[Ca^{2+}]_i$. The NPE trace suggests a very slight $[Ca^{2+}]_i$ increase. *B*, changes in $[Ca^{2+}]_i$ of both epithelial layers in response to phenylephrine. Upon exposure to phenylephrine, $[Ca^{2+}]_i$ in the PE rises from 0·1 to $0{\cdot}32 \pm 0{\cdot}7$ μM noise/fluctuation. Simultaneously, $[Ca^{2+}]_i$ in the NPE is slightly reduced by ~30%. *C*, representative trace obtained by confocal microscopy using fluo-3. As in the case of fura-2 ratiometric measurements in the slice preparation, there is a moderate increase in $[Ca^{2+}]_i$ in the PE. Due to the rapid decay of fluorescence it is not possible to establish unequivocally whether any small NPE $[Ca^{2+}]_i$ changes occur during this period.

the NPE peak by about 10 s (Figs 5a–d and 6*A*). The confocal approach allowed an additional important test, namely to characterize the responses of the NPE and PE cells within the physically complete CBE, after disconnecting the two layers by the standard technique (incubation in Ca^{2+}-free media) for physical separation of both layers from each other (Fain *et al.* 1988; Wolosin *et al.* 1993; Schütte *et al.* 1996). Following this treatment, the 'at rest' confocal images were indistinguishable from those obtained from control tissue. However, Ca^{2+} mobilization in response to α_2-adrenocholinergic input was now restricted to NPE cells (Fig. 6*B*; $n = 3$). Additionally, the onset of the Ca^{2+} rise in NPE cells no longer exhibited the usually observed synchronism. A comparative look at frames i, j and k (10 s apart) in Fig. 5 reveals that some cells start to respond substantially earlier than their neighbours (see also Fig. 6*C*). Attempts to study the pharmacological behaviour of the two layers following Ca^{2+}-free incubation in tissue slices were not feasible because the layers separated spontaneously during the mechanical cutting.

The complete lack of response of the disconnected PE to α_2-AR stimuli, whether after physical isolation from the NPE (Schütte *et al.* 1996), or even when in close proximity to the NPE (Fig. 6*B*) raises important questions regarding the nature and origin of the PE response in the intact CBE. It could be proposed that the incubation in Ca^{2+}-free medium irreversibly abolishes an existing α_2-AR pathway within the PE. This option is not particularly convincing, since other pharmacological PE mechanisms (e.g. cholinergic and α_1-AR responsiveness) are clearly preserved and more importantly, the α_2-adrenocholinergic synergism of the NPE is not affected. Considering the slight trailing of the PE mobilization with respect to those in the NPE and its lower level of synchronism, it appears more rational to assume that the PE activity in the intact tissue results from Ca^{2+} signal transfer from the NPE to the PE, as similarly described in other systems (Combettes, Tran, Tordjmann & Laurent, 1994; Hansen, Boitano, Dirksen & Sanderson, 1995).

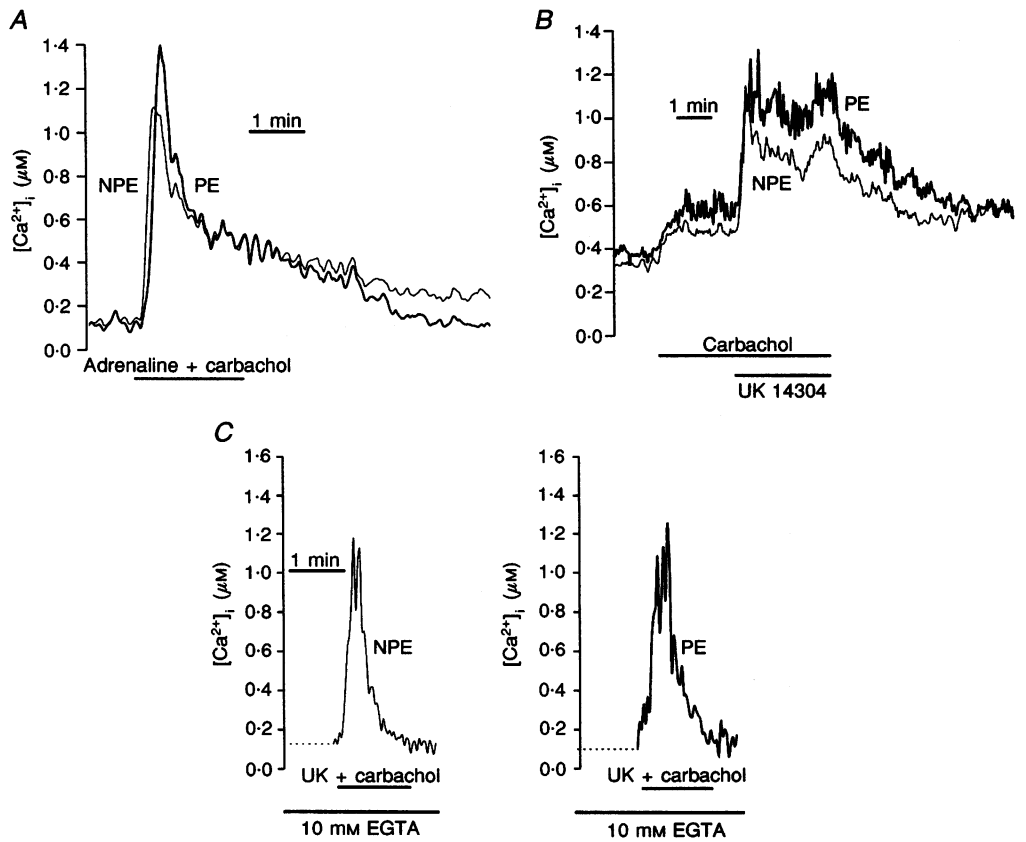

Figure 4. Simultaneous $[Ca^{2+}]_i$ responses of NPE and PE to adrenocholinergic stimuli

A, simultaneous addition of adrenaline and carbachol induces a dramatic Ca^{2+} mobilization in both epithelial layers. *B*, effect of sequential addition of carbachol and the α_2-AR-specific agonist UK 14304 (UK). The α_2-adrenocholinergic stimulus was maintained beyond the initial peak of the response. This resulted in a second $[Ca^{2+}]_i$ rise. *C*, effect of simultaneous exposure to UK 14304 and carbachol in EGTA (10 mM) following a 4 min pre-perfusion with EGTA (dotted line) to ensure complete removal of extracellular Ca^{2+}. In all cases NPE and PE responded almost simultaneously with similar amplitudes (for graphical clarity, the traces have been separated along the time axis). Note that while removal of extracellular Ca^{2+} had no apparent effect on the rising phase of the response, it caused a rapid decrease of $[Ca^{2+}]_i$ to sub-baseline values after passing the acme of the response.

One of the most obvious differences between the intact CBE and the Ca^{2+}-free treated tissue is the existence of cell-to-cell communications in the former. Thus, to explore the role of these gap junctions, we repeated the α_2-adrenocholinergic stimulation experiments in the presence of the junctional inhibitor heptanol (Bernardini, Peracchia & Peracchia, 1984; Wolosin, 1991) which, by itself, does not alter membrane permeability to Ca^{2+} (Bastide, Hérvé, Cronier & Délèze, 1995). Introduction of heptanol elicited a moderate decrease of $[Ca^{2+}]_i$ in the NPE cells (Fig. 7*A*). Upon withdrawal of heptanol, the NPE exhibited a rapid, often transiently overshooting $[Ca^{2+}]_i$ increase which reversed readily upon re-introduction of heptanol. We were able to quantify these $[Ca^{2+}]_i$ changes by *en face* fluorimetry for measurements of NPE $[Ca^{2+}]_i$ in the pigmented rabbit as described by Schütte *et al.* (1996); that is, withdrawal of heptanol following a 5 min exposure resulted in a 1·8-fold increase in $[Ca^{2+}]_i$ within 30 s ($n = 6$; not shown). The PE traces derived from the slice preparations suggested an increase in $[Ca^{2+}]_i$ with the introduction of heptanol.

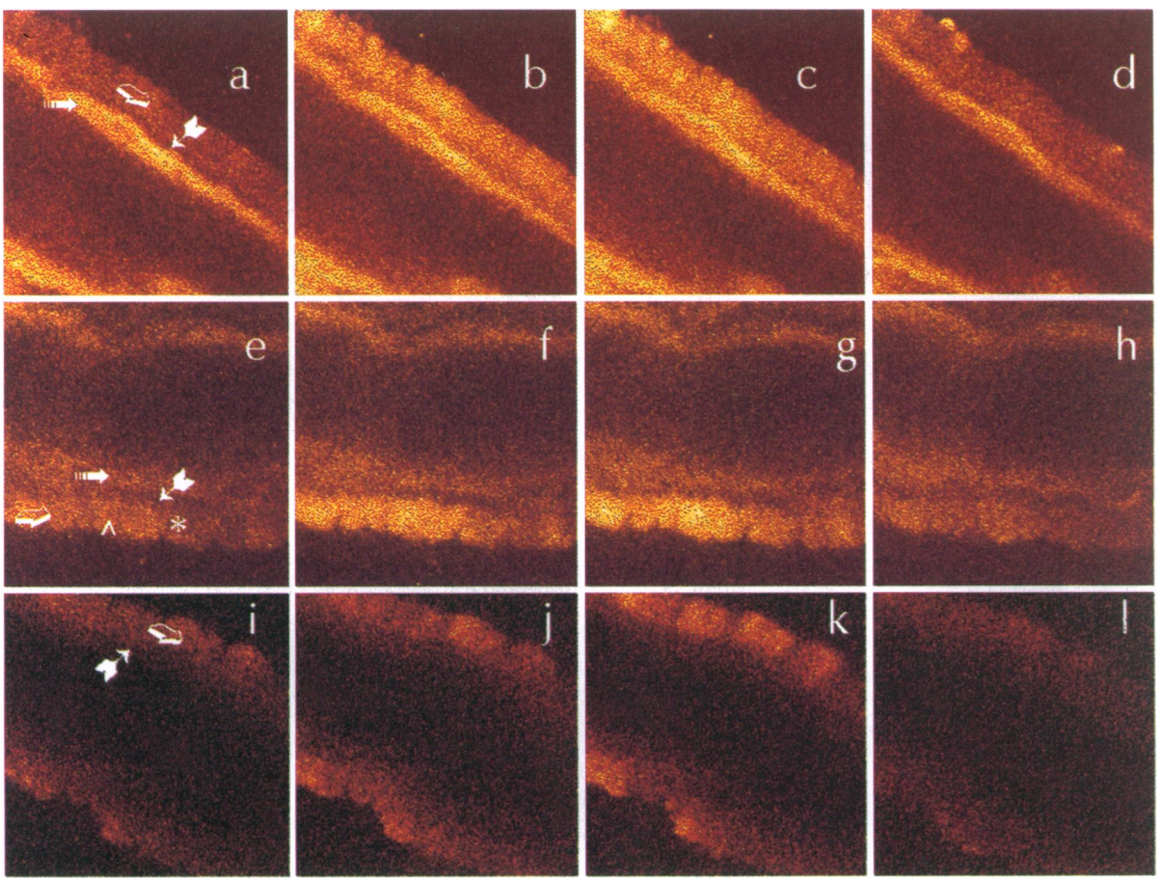

Figure 5. Sequential confocal frames showing the effects of adrenocholinergic stimuli on Ca^{2+}-induced changes in fluo-3 emission intensity

The pseudocolour scale 'amber glow' associates a change from red to yellow with brightness. The first three images in each row are directly consecutive (10 s apart) and show the last frame before the onset of the response, the onset of the response and the peak of the response. The last frame in each row shows the return to baseline values, typically 90 s after the peak. Frames a–d, control experiment. Prior to stimulation (frame a), the NPE (⇨) has a starting value slightly higher than the PE (➠). The brightest fluorescence is seen in the endothelial layer (➡). Upon stimulation with UK 14304 and carbachol, both NPE and PE respond in full synchronism with a massive (*ca* ×2·5) increase in fluorescence (frames b and c). Ninety seconds after reaching the acme of the response, $[Ca^{2+}]_i$ has returned to baseline values in both layers (frame d). Endothelial cells do not respond to the adrenocholinergic stimulation. Frames e–h, heptanol- induced inhibition of gap junctional communication. Note that the NPE responds strongly to adrenocholinergic stimulation, while no response occurs in the PE. The two individual cells marked by Λ and ∗ were used to generate the data represented in Fig. 7. The cell which shows the 'brighter' response (Λ) actually has the smaller increase in fluorescence emission compared with the cell marked by ∗ which is labelled less intensely but has a larger relative Ca^{2+} response (see Fig. 7*E*). Frames i–l, response to adrenocholinergic stimuli after breakdown of gap junctions by Ca^{2+} deprivation. Note that only the NPE responds with an increment of fluorescence (frames j and k). This experiment was run at a lower intensity of the laser which is modified in the darker, redder colours of the cells.

However, these apparent changes were too small, *vis-à-vis* the magnitude of the noise and/or spontaneous fluctuations, to permit an unequivocal determination.

Despite its effect on $[Ca^{2+}]_i$ baseline values, the presence of heptanol in the perfusion medium had no visible effect on the NPE response to the combined α_2-adrenocholinergic stimulation. The PE response, on the other hand, was modified. The modified responses could be grouped, subjectively, into three distinct patterns. In twelve experiments (5 by ratiometry and 7 by intensitometry) the onset of the PE $[Ca^{2+}]_i$ was delayed by 10–20 s with respect to the onset of that in the NPE, but the amplitude of the response was not visibly affected (Fig. 7*B* and *C*). In two intensitometric experiments, the onset of the PE response trailed with respect to the NPE and additionally the magnitude of the response underwent a substantial decrease (Fig. 7*D*), although it was clearly larger than could be expected from the effect of carbachol alone on the PE. In four experiments the PE response was either null or was small enough to be caused by the cholinergic agonist itself (Figs 5e–h and 7*E*). The one consistent feature of all experiments using heptanol was that 10 s after the onset of the NPE response, there was no detectable $[Ca^{2+}]_i$ increase in the PE cells. This is in sharp contrast to the control experiments, in which 10 s were usually enough to bring the PE $[Ca^{2+}]_i$ close to its peak. Objectively, as discussed below, the different patterns may reflect a continuous range of the heptanol-induced effect on the Ca^{2+} mobilization signal transference, resulting from differences in the extent of baseline cell-to-cell communication levels between individual samples.

DISCUSSION

The results of the studies of Ca^{2+} mobilization in response to adrenocholinergic stimuli performed in both the physically and the optically dissected ciliary body are mostly consistent with the results of our previous studies of the isolated NPE and PE layers. In the NPE, carbachol and brimonidine produced small responses by themselves and a large Ca^{2+} mobilization when combined with each other, whereas phenylephrine had no effect. Since neither of the methodologies employed in the present study could have altered the pharmacological profile of NPE cells in the intact CBE as reported earlier (Farahbakhsh & Cilluffo, 1994; Schütte *et al.* 1996) these results could have been expected. Also the fact that in the PE, carbachol and phenylephrine each elicited a moderate Ca^{2+} response could have been predicted from the results obtained from dissociated epithelial layers (assuming that the dissociation had no effect on the pharmacological profile of the cells). The one novel finding of the present study is the rapid, massive Ca^{2+} mobilization occurring within the PE, which parallels the synergistic response of the NPE upon adrenocholinergic stimulation. The absence of such a brimonidine effect in the dissociated tissue suggests that this particular pattern of Ca^{2+} mobilization is not endogenous to the PE but rather stems from the extensive gap junctional coupling between both epithelial layers. This notion is supported by the effect of heptanol, a well-known inhibitor of junctional communication. In the NPE, heptanol itself had little effect on the response characteristics, but in the PE, its presence modified the responses by either: (1) inducing a marked delay, with or without a decrease in amplitude; or (2) completely

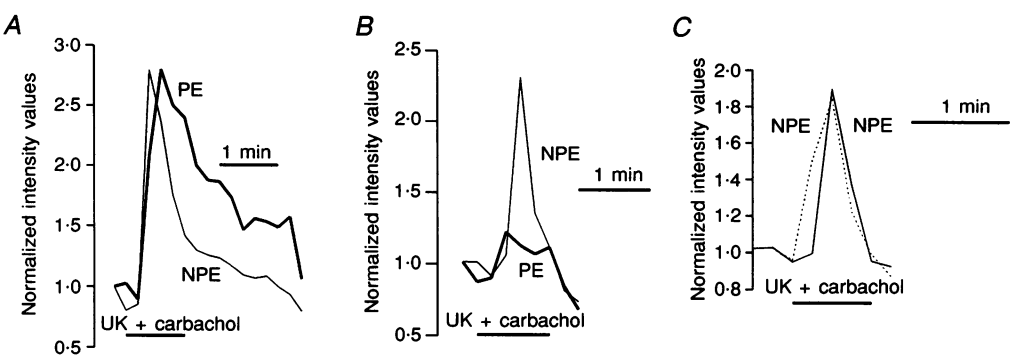

Figure 6. Effect of junctional communication breakdown on the magnitude and synchronism of adrenocholinergic responses

A, control experiment using the optical slice obtained by confocal microscopy and fluo-3 intensitometry. This experiment was run in ×4 oversampling mode. Both PE and NPE appear to respond to α_2-adrenocholinergic stimulation in unison with a slight (10 s) delay of the PE response peak. Since the temporal resolution of the acquisition system is one data point every 10 s, the actual response peaks may not necessarily coincide with the values shown. *B*, after incubating the ciliary body in Ca^{2+}-free Ringer solution at 37 °C for 150 min, which results in breakdown of the heterocellular gap junctions, the NPE shows a normal response to simultaneous UK 14304 and carbachol exposure. The PE response is, however, almost completely blocked under these conditions, a small residual increase in intensity is likely to be caused by carbachol alone. *C*, breakdown of gap junctional communication also disrupts synchronicity of the NPE. The two traces show Ca^{2+} responses of two neighbouring NPE cells, which are desynchronized by about 10 s.

abolishing the large Ca^{2+} response of the PE, except for a small $[Ca^{2+}]_i$ increase attributable to the effect of carbachol itself.

Bernardini *et al.* (1984) found 3 mM heptanol to cause a maximal 96% inhibition of the junctional communication in *Xenopus* embryos. Considering the molecular diversity of the connexin family, one has to expect a variable degree of inhibition in different systems, therefore, the maximal inhibition of the NPE–PE junctions may be smaller or larger than that. Under control conditions, NPE and PE responses are maximally synchronized, i.e. the longest delay we observed was in the order of 2 s for the onset of the responses, consistent with the rapidity of transfer observed in other systems (Combettes *et al.* 1994; Hansen *et al.* 1995). At this moment, the NPE response itself is still in its initial phase, i.e. below 10% of its maximal amplitude. If one assumes that the kinetics of the signal transfer messengers are reflected in the $[Ca^{2+}]_i$-change kinetics, the amount of signal necessary to mount a response in the PE might still be reached at the apex of the NPE response, even when as much as 90% of the heterocellular junctional permeability has been abolished. Under these conditions, the initiation of the PE response will occur when the increased amount of messenger compensates for the reduced communication between both layers. In some experiments, a stronger junctional inhibition in conjunction with a lower baseline communication level and/or a weaker messenger build-up in the NPE might produce a state in which the threshold for transfer of a response is not achieved altogether.

Ca^{2+} signals generated in the PE by exposure to α_1-AR agonists do not seem to be reflected in NPE $[Ca^{2+}]_i$. Therefore, it appears that signal transfer between both layers of the CBE is only unidirectional, occurring from the NPE to the PE but not vice versa, i.e. in a rectified manner. Rectification across gap junctional paths between individual units of heterocellular complexes has been shown in a variety of invertebrate (Furshpan & Potter, 1959; Nicholls & Purves, 1972; Giaume & Korn, 1983, 1984; Margiotta &

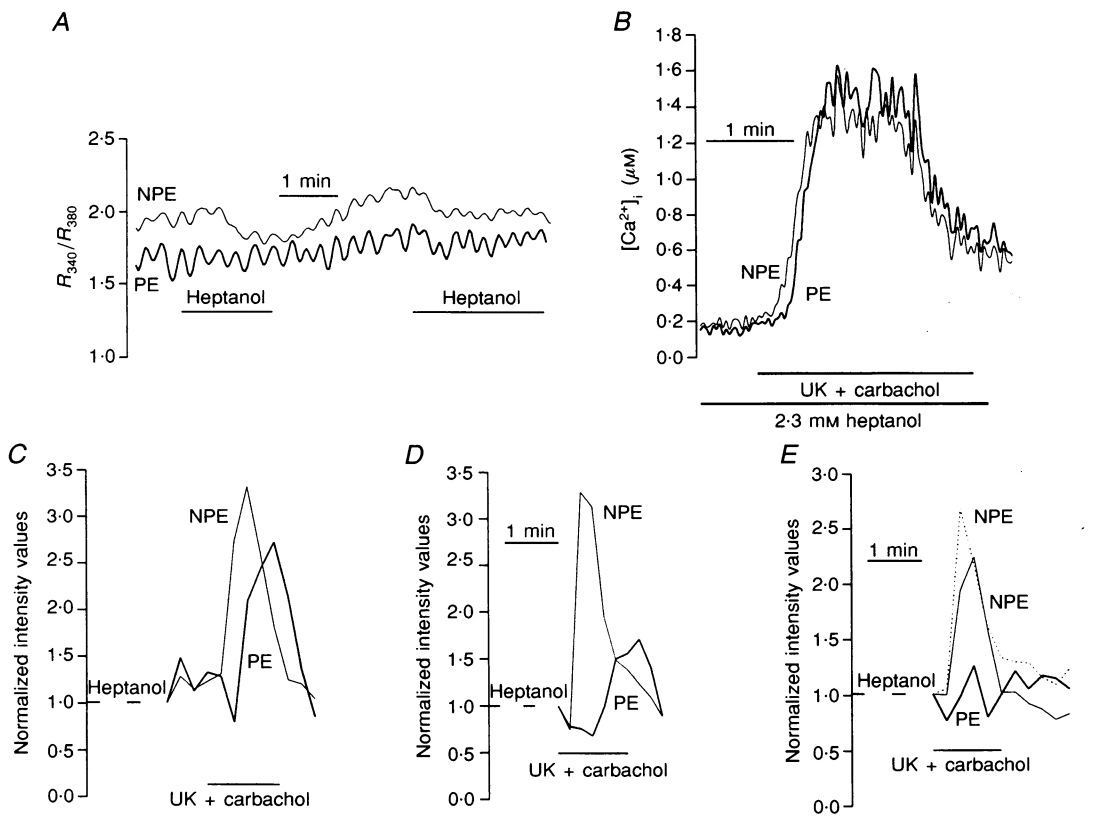

Figure 7. Effect of heptanol on baseline $[Ca^{2+}]_i$ and on the adrenocholinergic response

A, effect of 3·5 mM heptanol on baseline $[Ca^{2+}]_i$ in both layers of the CBE. Note that the NPE ratio decreases reversibly once heptanol is introduced. In the PE, heptanol has no such drastic effect, but can lead to a slight, sometimes delayed increase in baseline ratio. *B*, effect of 2·3 mM heptanol on the response to UK 14304 and carbachol. Note the delayed start of the PE response which, once it is activated, exhibits a normal course. *C–E*, fluo-3-intensitometry; variable effects of 3·5 mM heptanol on the PE responses. *C*, heptanol causes a small (~10 s) delay of the response onset. *D*, heptanol causes a more than 20 s delay of the PE response in conjunction with a reduction of the PE peak response amplitude. *E*, heptanol blocks the transmission of the synergetic NPE response to the PE. The dotted line represents the intensity changes in the cell marked with * and the continuous thin line the response of the cell marked with Λ in Fig. 5e. In the PE no response above noise can be detected.

Walcott, 1983) and vertebrate (Ringham, 1975) systems and, therefore, is not necessarily surprising. Any substantive elaboration on plausible mechanisms in the CBE system is currently hindered by our limited knowledge on the nature of the intracellular mechanisms that converge to the generation of the dramatic synergetic response within the NPE. Nevertheless, the majority of intracellular Ca^{2+} stores follows a rather conservative layout regarding receptor identity; all cells possess intracellular Ca^{2+}-sequestering compartments sensitive to IP_3 (Assmann & Wu, 1994; Danoff & Ross, 1994; Ehrlich, Kaftan, Bezprozvannaya & Bezprozvanny, 1994). Therefore, Ca^{2+} stores in the PE may be sensitive to the same messenger(s) as those in the NPE and diffusion of these messengers across gap junctions could readily trigger the release of Ca^{2+} from intracellular PE compartments (Hansen *et al.* 1995). In this regard, the obvious differences between the amount of cytoplasmic space in NPE and PE cells may be critical. The latter are much smaller (as low as one-third of the NPE volume in the rabbit; see Figs 1*A* and 2*B*) and their cytoplasm is literally filled with granules. A morphometric analysis of our own electronmicrographs (Wolosin *et al.* 1993) indicated that pigment granules occupy more than one-third of the total cellular space of the rabbit PE cells (M. Schütte and J. M. Wolosin, unpublished observation). Thus, the PE:NPE cytoplasmic volume ratio may be as small as 1 : 4·5. A rise in the concentration of any second messenger in the NPE could therefore easily affect the PE whereas the much larger amount of NPE cell cytoplasm may act as a sink for messengers originating in the PE and thus maintain the signal below activation threshold. If one allows for any diffusion between both epithelial layers, it is conceivable that Ca^{2+} mobilization in the PE may be better effected by messengers originating in the NPE than from its own (α_1-adrenoceptor-induced) mechanisms, especially since this path lacks the capability to synergistically interact with the cholinergic system.

In addition to the cytoplasmic volume, the kinetics of the response in each cell type have to be considered. During the synergetic adrenocholinergic response, $[Ca^{2+}]_i$ rises to levels much higher than those attainable in the PE by phenylephrine stimulation (> 1·5 μM *vs.* ~0·3 μM) and the rate of $[Ca^{2+}]_i$ rise is 10- to 100-fold faster in the NPE (Schütte *et al.* 1996). Furthermore, these differences in rates and concentrations are based on averages over whole cells. Given the wave-like spreading nature of Ca^{2+} mobilization which can involve local 'hot spots' (von Gersdorff & Matthews, 1994), for a brief moment the concentration of mediators within the vicinity of the apical domain in the NPE may also be much higher than the average concentration. Such a phenomenon may underpin the surprising resiliency of the signal transmission to heptanol treatment. Even if heptanol decreases junctional permeability to less than 10% of control, a very large local messenger gradient may overcome the artificial restriction. The restriction will be reflected only in a delay in the onset of the transmission. This is not likely to be the case in the PE where the slow $[Ca^{2+}]_i$ build up (Schütte *et al.* 1996; also compare Fig. 3 with Fig. 4) should allow quasi-homogeneity throughout the cell. Thus, the effective differences in mediator gradients may be much larger than those inferred from the whole-cell measurements and NPE messengers may easily reach threshold levels for activation in the PE whereas the inverse will not occur.

Electrical (Margiotta & Walcott, 1983; Giaume & Horn, 1983, 1984), ionic (Peracchia & Peracchia, 1980*a*, *b*) or pharmacological (Teranishi, Negishi & Kato, 1983; Piccolino, Neyton & Gerschenfeld, 1984; Lasater & Dowling, 1985; Hampson, Vaney & Weiler, 1992; Peracchia, 1993; Mills & Massey, 1995) modulations need to be considered, too. The α_2-AR and/or α_1-AR mechanisms by themselves may affect the baseline junctional NPE:PE permeability (Oh, Krupin, Tang, Sveen & Lahum, 1994; Edelman *et al.* 1994) enhancing or hindering signal transmission. The recently demonstrated partial inhibition of the NPE:PE junctional path (~50%) by α_1-AR agonists (Shi, Wolosin & Candia, 1996), is fully consistent with this notion.

In this context, the intriguing effect of heptanol on baseline NPE $[Ca^{2+}]_i$ is noteworthy. The aqueous humour contains precisely controlled levels of Ca^{2+}. Jacob (1991) has shown that only the PE possesses Ca^{2+} channels and suggested that like other solutes, Ca^{2+} may be transported from the PE to the NPE through the heterocellular path en route to the aqueous humour. The effect of heptanol supports such a proposal. A substantial inhibition, barring activation of other regulatory mechanisms of the junctional path (Shi *et al.* 1996) should modify the steady-state levels in both cells, downwards in the NPE and upwards in the PE. Upon removal of heptanol the NPE $[Ca^{2+}]_i$ should be re-established, which is consistent with our observations. Moreover, exposure to heptanol may induce insertion of new gap junctions into the plasma membrane to compensate for the heptanol-induced inhibition (Bernadini *et al.* 1984). This may explain the rapidity of the return to baseline $[Ca^{2+}]_i$ levels after heptanol removal.

The effect of the synergetically induced Ca^{2+} mobilization on either the overall secretory activity of the CBE or in specific transport events remains unknown as yet. Topically applied α_2-AR agonists are potent antisecretory agents but their site and mechanism of action remains a matter of speculation (Toris, Gleason, Camras & Yablonski, 1995). However, irrespective of the specific impact of Ca^{2+} mobilization on aqueous humour production, our data suggest strong differences regarding the roles of the two cell layers in the overall pharmacological control of the tissue. While the NPE can set the physiological tone for the whole CBE, including the PE, responses of the latter remain confined to their site of origin. Our results also demonstrate the potential intricacies of gap junctionally mediated communication in a heterocellular system. It is tempting to further associate the differential effects of α_1- and α_2-adrenoceptor ligands on aqueous humour production with their differential effects on

$[Ca^{2+}]_i$ reported in our study. However, more work will be necessary, to deepen our understanding of the possible systemic impact of the synergetic adrenocholinergic response, before the complexity of this issue can be resolved.

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