Synergistic effect of adrenergic and muscarinic receptor activation on $[Ca^{2+}]_i$ in rabbit ciliary body epithelium

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1. Changes in cytosolic free calcium concentration ($[Ca^{2+}]_i$) in response to cholinergic and adrenergic agents alone and in combination were investigated using fura-2 fluorescence imaging in intact non-pigmented epithelial cells of rabbit ciliary body.

2. Resting ('baseline') $[Ca^{2+}]_i$ was 147 ± 6 nM (mean ± S.E.M.). Acetylcholine (ACh, 10 μM) doubled $[Ca^{2+}]_i$, and adrenaline (1 μM) increased it by about 36 %. When ACh (10 μM) and adrenaline (1 μM) were applied together $[Ca^{2+}]_i$ was transiently increased to 1160 ± 160 nM, about 7 times the response induced by ACh alone.

3. Noradrenaline and 5-bromo-6-(2-imidazolin-2-yl-amino)-quinoxaline (UK 14304) had effects similar to adrenaline in enhancing the response to ACh. Phenylephrine (Phe) had a relatively smaller effect and none was observed for methoxamine and isoprenaline (Iso).

4. The response to ACh and adrenaline could be blocked by atropine (1 μM, 87 ± 5 %), yohimbine (1 μM, 73 ± 8 %), and to a lesser degree by prazosin (1 μM). Propranolol had no effect.

5. Lowering the extracellular calcium concentration to 3 nM dropped the baseline $[Ca^{2+}]_i$ by half and reduced the response to ACh and adrenaline to a small and transient rise in $[Ca^{2+}]_i$. Addition of La^{3+} to Ca^{2+}-containing solution also lowered $[Ca^{2+}]_i$ and largely reduced the response.

6. We conclude that simultaneous activation of muscarinic and α_2-adrenergic receptors induces a large increase in $[Ca^{2+}]_i$, which is the result of both Ca^{2+} release and influx.

Ciliary body epithelium is considered responsible for the secretion of aqueous humour in the eye, but the regulatory mechanisms of aqueous humour formation are not yet fully understood (Brubaker, 1991). Though there is no evidence for direct neural innervation of ciliary body epithelial cells, both sympathetic and parasympathetic nerves have been observed within the ciliary processes in the vicinity of the epithelial cells (Stone, Kuwayama & Laties, 1987). Furthermore, agents synthesized in the eye and circulating hormones such as adrenaline may reach these cells by diffusion (Elayan, Kennedy & Ziegler, 1990).

Since the ciliary body epithelium appears not to receive direct synaptic input, and since there is such a multitude of receptors present on these cells (see Wax, 1992), it seemed to us possible that the epithelial cells may be regulated by the combined effect of multiple receptor activation, as has been suggested for platelet aggregation (Ruffolo, Nichols, Stadel & Hieble, 1993). In this report, we show such an effect for combined application of muscarinic and adrenergic agonists. Whereas ACh and adrenaline applied by themselves each produce small increases in $[Ca^{2+}]_i$, simultaneous application produces an over 7-fold increase, in part as a result of Ca^{2+} influx across the plasma membrane. This influx appears to occur through a La^{3+}-sensitive entry pathway.

METHODS

Tissue isolation

Intact ciliary body epithelial processes were isolated from pigmented rabbits by the following procedure. Rabbits weighing 2–3 kg were killed with a lethal dose of sodium pentobarbitone (200 mg). The eyes were then rapidly enucleated, collected and rinsed in Hepes-buffered Ringer solution (for formulation, see below). The anterior segments were isolated, pinned (cornea down) in a dissecting dish filled with Ringer solution, and the lens and lens capsule carefully removed. Single processes were sectioned from the ciliary body by cutting along the base of the process from the iridial margin to pars plana. Individual processes were laid on their sides in Ringer solution in 35 mm Petri dishes that had been modified by cementing a glass coverslip over a 13 mm hole drilled into the bottom of the dish. The processes were barely covered and held down with a 2–3 mm square piece of glass coverslip, to improve mechanical stability.

A plexiglass insert was placed in the chamber to reduce the chamber volume. The process was continuously superfused

with Ringer solution or a test solution through a gravity-fed system at a rate of 5–10 ml min^{-1}. Solutions were evacuated from the chamber by suction.

Solutions

Hepes–Ringer was of the following composition (mM): 137 NaCl, 4·3 KCl, 1·7 $CaCl_2$, 0·8 $MgCl_2$, 10 sucrose, 7 glucose, 10 Hepes, 6 NaOH (pH 7·6, 293–298 mosmol l^{-1}). Low-calcium Ringer was prepared by substituting 10 mM EGTA for equiosmolar NaCl (calculated extracellular calcium concentration, $[Ca^{2+}]_o$, < 3 nM). Adrenaline, noradrenaline, carbachol, propranolol, yohimbine, methoxamine, oxymetazoline and UK 14304 were purchased from Research Biochemicals, Inc. (Natick, MA, USA); prazosin, lanthanum, clonidine, phenylephrine, isoprenaline and ionomycin were from Sigma Chemical Co. (St Louis, MO, USA); and acetylcholine was from Receptor Research Chemicals, Inc. (Baltimore, MD, USA). All drugs were prepared as concentrated stocks and stored at −20 °C.

Fura-2 loading and Ca^{2+} imaging

Ciliary processes were loaded with 10 μM fura-2 acetoxymethyl ester (fura-2 AM; Molecular Probes, Inc., Eugene, OR, USA) for 30–120 min at room temperature and then washed with Ringer solution. $[Ca^{2+}]_i$ measurements were made with an imaging system composed of an inverted microscope (IM, Zeiss, Oberkochen, Germany), an image intensifier (KS-1381, Videoscope, Sterling, VA, USA), a CCD camera (CCD72, DAGE-MTI, Michigan City, IN, USA) and a filter wheel of conventional design. The filter wheel and intensifier gains were controlled by computer (RS20, Hewlett-Packard, Sunnyvale, CA, USA).

The emitted fluorescence (510 nm) resulting from excitation at 340 and 380 nm was measured as follows. The fluorescence images were digitized using an image acquisition board (DT 2681, Data Translation, Marlboro, MA, USA), and the images were displayed on a video monitor (PVM-1221Q, Sony). The average intensity of an area encompassing four to five non-pigmented cells was measured and used for calculation of the emission ratio. The emission ratio was plotted against time on a computer monitor. Ca^{2+} concentration was calculated from the ratio of emission intensities using the method of Grynkiewicz, Poenie & Tsien (1985). The saturating level of $[Ca^{2+}]_i$ was determined *in situ* after perfusion of the process with 5 μM ionomycin in Hepes–Ringer solution. To determine the minimum level of $[Ca^{2+}]_i$, the process was perfused with low-calcium Ringer solution. We used the method of Owen (1991) to estimate the error in calculating $[Ca^{2+}]_i$. Only the results of experiments in which relative error remained below 15 % were used.

RESULTS

ACh and adrenaline increase $[Ca^{2+}]_i$

The baseline $[Ca^{2+}]_i$ level recorded from intact non-pigmented ciliary body cells was 147 ± 6 nM (mean ± S.E.M., $n = 329$). Application of 10 μM ACh by itself caused an increase in the $[Ca^{2+}]_i$ to 290 ± 23 nM or 117 ± 19 % ($n = 92$) over baseline (see Fig. 1, bar 'ACh'). In addition, we observed a small increase in $[Ca^{2+}]_i$ (36 ± 13 %, $n = 3$) due to

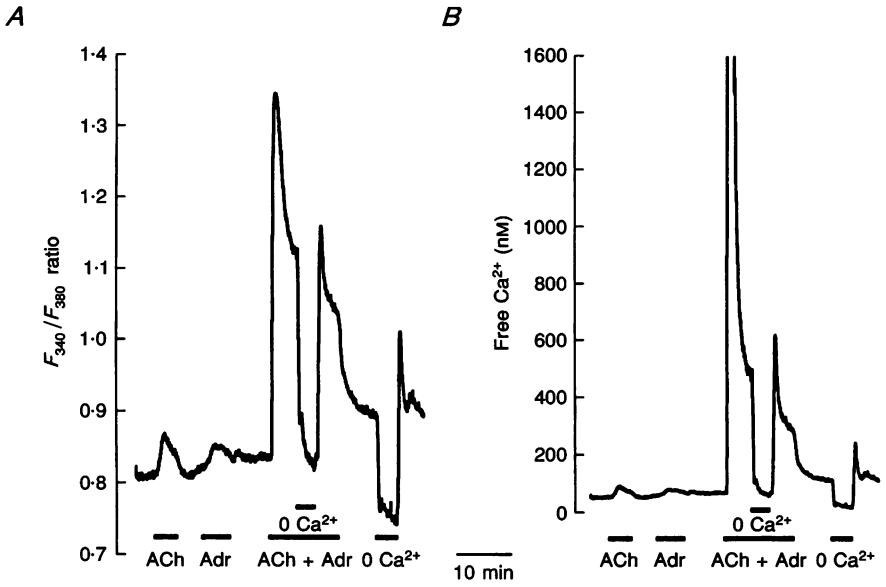

Figure 1. Increase in intracellular Ca^{2+} concentration in response to ACh and adrenaline in intact ciliary body epithelial cells

A, plot of emission ratio *versus* time. *B*, same experiment as in *A* but calibrated to indicate $[Ca^{2+}]_i$ and drawn on an expanded scale. *A* and *B*, application of either ACh (10 μM) or adrenaline (Adr, 1 μM), shown by bars, caused a small increase in the $[Ca^{2+}]_i$. However, when ACh and adrenaline were applied together, the $[Ca^{2+}]_i$ rose dramatically to micromolar levels. The response is composed of a large transient component followed by a decline to a sustained but still elevated level of intracellular Ca^{2+}. Exposure to low-Ca^{2+} Ringer solution (0 Ca^{2+}) caused a rapid and reversible drop in the elevated $[Ca^{2+}]_i$ elicited by the combined drug application. Due to the inherent inaccuracy in the calibration of the high $[Ca^{2+}]_i$ values seen in the peak of the ACh/adrenaline response, the Ca^{2+} concentration was not determined above the level of 1600 nM.

the application of 1 μM of the adrenergic agonist adrenaline (Fig. 1, bar 'Adr'). These data confirm the observations of others on cultured ciliary body non-pigmented cells (Lee, Reisine & Wax, 1989; Ohuchi, Yoshimura, Tanihara, Kuriyama, Ito & Honda, 1992; Crook & Polansky, 1992).

When the epithelium was simultaneously exposed to ACh and adrenaline, a much larger increase in $[Ca^{2+}]_i$ was observed. This can be seen above the bar labelled 'ACh + Adr' in Fig. 1. For this figure, we show to the left the 340/380 nm ratio, and to the right the change in $[Ca^{2+}]_i$ calculated from the 340/380 nm ratio and the *in situ* calibration. Notice that the $[Ca^{2+}]_i$ in Fig. 1*B* is shown for only a part of the data, since the increase in $[Ca^{2+}]_i$ at its peak was so large that the calibration of fura-2 was no longer accurate enough to give a meaningful value for the $[Ca^{2+}]_i$. For the subset of experiments for which the calibration could be used for the peak of the response (relative error < 15 %), application of 10 μM ACh and 1 μM adrenaline caused a transient increase of the $[Ca^{2+}]_i$ to an average peak value of 1160 ± 160 nM (or 1497 ± 296 %, $n = 50$). This value is likely to be an underestimate, since we have excluded the much larger increases like those in Fig. 1. After the initial peak increase, $[Ca^{2+}]_i$ declined to a sustained but elevated level, which averaged 434 ± 36 nM (529 ± 77 %). Removal of the drugs caused the $[Ca^{2+}]_i$ to decline, though in some cases (as in Fig. 1) the $[Ca^{2+}]_i$ never fell back to the level before the application. Similar but slightly smaller effects were observed when noradrenaline was used in place of adrenaline: noradrenaline (1 μM) alone raised $[Ca^{2+}]_i$ by 18 ± 13 % ($n = 4$) over the baseline, and the combined application of ACh and noradrenaline increased $[Ca^{2+}]_i$ by 973 ± 332 %.

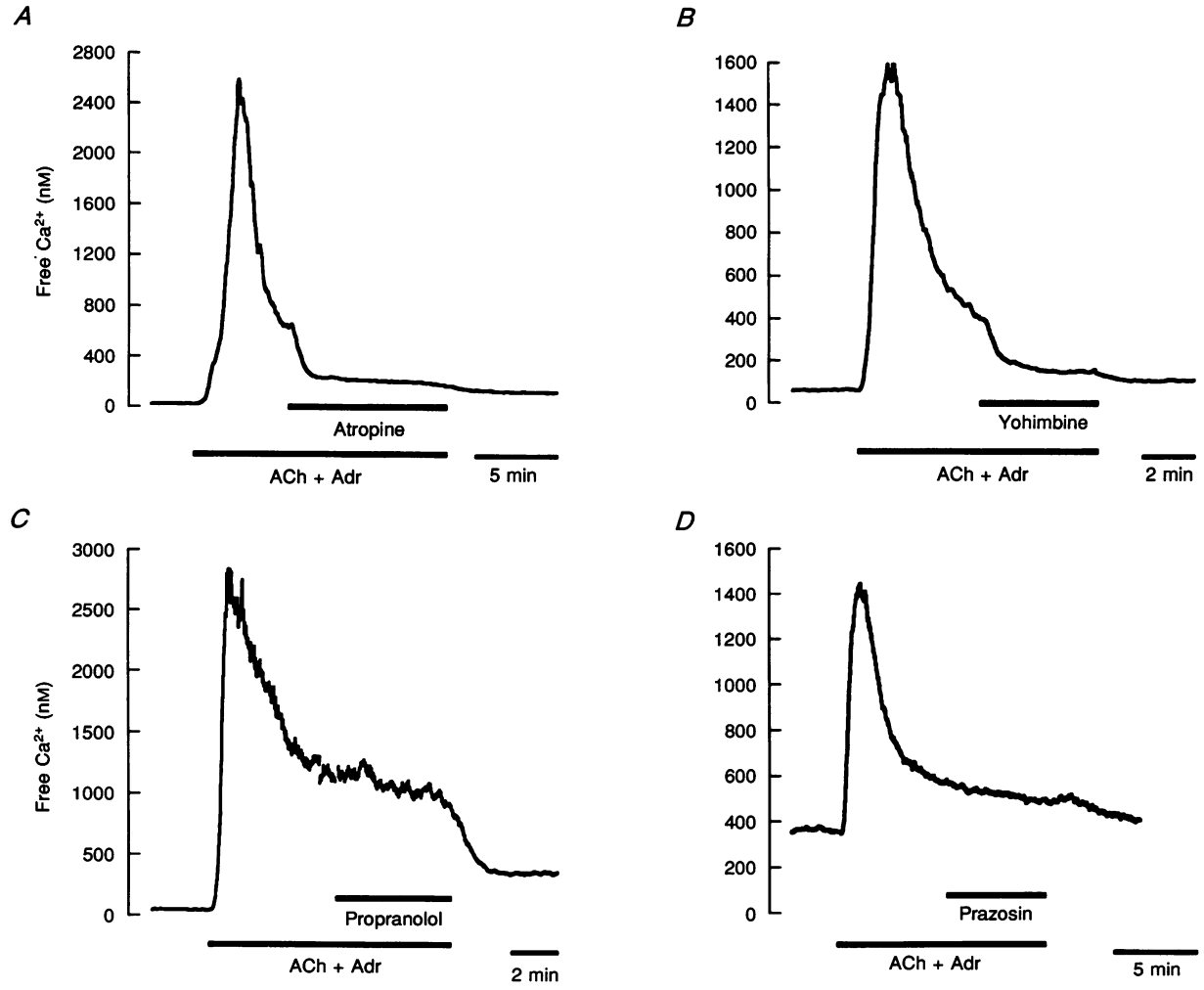

Figure 2. The effect of muscarinic and adrenergic antagonists on the response induced by 10 μM ACh and 1 μM adrenaline

Atropine (1 μM, *A*), a muscarinic cholinergic antagonist, and yohimbine (1 μM, *B*), an α_2-antagonist, substantially lowered the sustained level of intracellular Ca^{2+} elicited by the combined drug application. However, application of the β-antagonist propranolol (1 μM, *C*) or the α_1-antagonist prazosin (1 μM, *D*) did not significantly affect the response.

Increase in $[Ca^{2+}]_i$ is mediated by muscarinic and α_2-adrenergic receptors

The cholinergic component of the increase in $[Ca^{2+}]_i$ induced by ACh and adrenaline was due to the activation of muscarinic receptors, since it was blocked (87 ± 5 %, $n = 8$) with 1 μM atropine (Fig. 2*A*). In order to characterize the contribution of the adrenergic component of the response, we applied several adrenergic antagonists to the tissue. Figure 2*B–D* show the effect of these agents on $[Ca^{2+}]_i$ after simultaneous exposure to ACh and adrenaline. Application of 1 μM of the α_2-antagonist yohimbine (Fig. 2*B*) substantially (73 ± 8 %, $n = 5$) and reversibly blocked the response to simultaneous ACh and adrenaline application. However, 1 μM propranolol, a β-antagonist, did not significantly decrease the sustained level of $[Ca^{2+}]_i$ (Fig. 2*C*, $n = 3$), and the α_1-antagonist prazosin (1 μM, Fig. 2*D*) had only a small and variable effect (37 ± 10 %, $n = 7$).

Further verification of the predominant α_2 nature of the adrenergic component was demonstrated with the use of the specific α_2-agonist UK 14304. This is shown in Fig. 3*A*. Although 1 μM UK 14304 alone in fact caused a discernable drop in the $[Ca^{2+}]_i$ (to 86 ± 4 % of the baseline, $n = 4$), UK 14304 added with 10 μM ACh mimicked the response seen with ACh and adrenaline. On average, the combination of ACh and UK 14304 increased the $[Ca^{2+}]_i$ to a peak 1740 ± 730 % ($n = 15$) of the baseline. However, the non-selective α-adrenergic agonist phenylephrine (Phe, 1 μM, Fig. 3*B*), while having no significant effect on its own (2·2 ± 11·4 %, $n = 4$), only moderately enhanced the effect of ACh on $[Ca^{2+}]_i$ (a 372 ± 87 % rise over the baseline, $n = 4$). The β-agonist isoprenaline (1 μM, Fig. 3*C*) and α_1-agonist methoxamine (2–75 μM, not shown) failed to show any significant effect when applied alone (7·9 ± 4·5 %, $n = 6$, and 1·5 ± 3·8 %, $n = 5$, respectively), nor did they enhance the response to ACh (95 ± 30 %, $n = 6$, and 52 ± 27 %, $n = 3$, respectively). These experiments demonstrate that the adrenergic component of the response was predominantly α_2 in nature.

Extracellular Ca^{2+} is required for the large increase of $[Ca^{2+}]_i$ in response to ACh and adrenaline

When during stimulation with ACh and adrenaline the extracellular Ca^{2+} was lowered by the perfusion of the process with low-calcium Ringer solution, the intracellular Ca^{2+} concentration rapidly fell to basal levels (see Fig. 1, bars labelled '0 Ca^{2+}'). When the external Ca^{2+} was returned to normal (with the process still exposed to ACh and adrenaline), the $[Ca^{2+}]_i$ returned to the previous level after a brief overshoot.

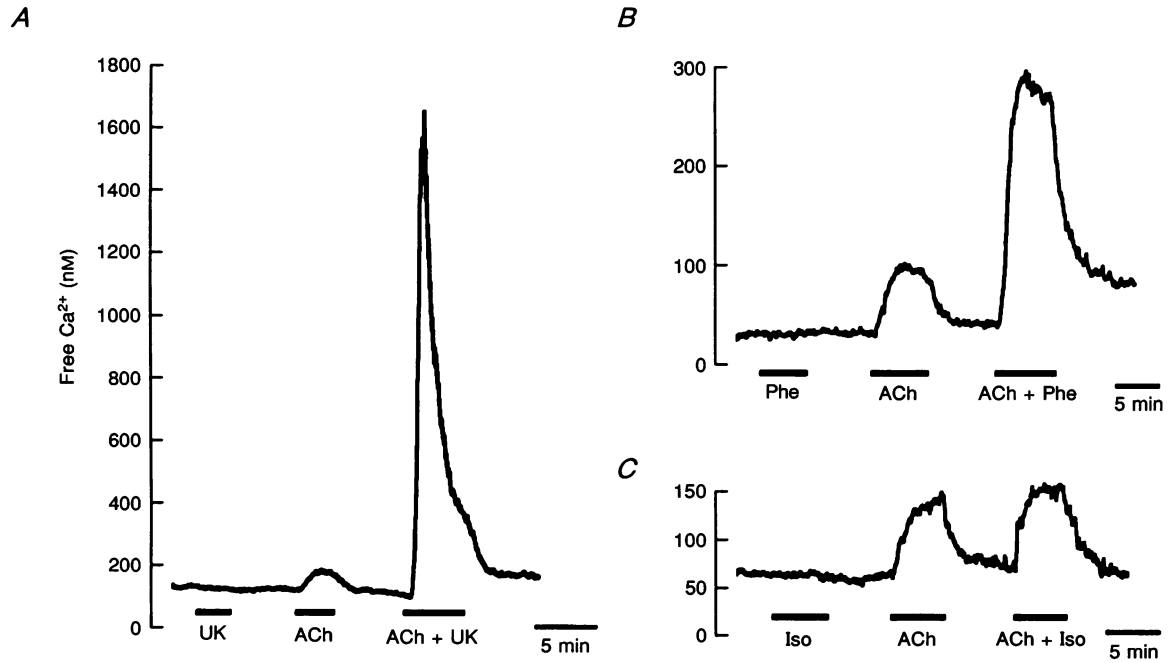

Figure 3. The adrenergic component of the combined ACh and adrenaline response is due to an α_2-receptor subtype

A, a specific α_2-agonist, UK 14304 (UK, 1 μM) did not cause a discernable increase in the $[Ca^{2+}]_i$. However, application of UK 14304 and ACh together (ACh + UK), mimicked the response elicited by adrenaline and ACh. Like UK 14304, phenylephrine (Phe, 1 μM, *B*), a non-specific α-agonist, and isoprenaline (Iso, 1 μM, *C*), a β-agonist, did not increase the $[Ca^{2+}]_i$ when applied alone. However, these agonists had little or no enhancing effect on the increase in $[Ca^{2+}]_i$ induced by ACh (*B*, ACh + Phe; *C*, ACh + Iso).

Exposure of the ciliary body epithelial cells to low-calcium Ringer solution led to a $49 \pm 4\%$ ($n = 14$) drop in the baseline $[Ca^{2+}]_i$ (Fig. 4*A*). Under these conditions, application of ACh and adrenaline together induced only a small and transient response ($203 \pm 20\%$ of the baseline *in the low-calcium Ringer solution*), which lasted only 113 ± 9 s ($n = 7$) even though the agonists were still present in the medium. The response to ACh and adrenaline could also be reduced by addition of low concentrations of La^{3+}. Lanthanum (1 μM) lowered the baseline $[Ca^{2+}]_i$ by $33 \pm 4\%$ ($n = 8$, Fig. 4*B*). In the presence of La^{3+}, ACh and adrenaline induced only a transient response (Fig. 4*B*, $[Ca^{2+}]_i$ rose by $400 \pm 75\%$ of the baseline *in the La^{3+}-containing Ringer solution*, $n = 5$). Furthermore, in a separate group of experiments, when 1 μM La^{3+} was applied after the response had reached the sustained level, the $[Ca^{2+}]_i$ rapidly fell to near the baseline (Fig. 4*C*, $82 \pm 19\%$ block, $n = 3$). The effect of La^{3+} was reversible. However, Cd^{2+} (up to 100 μM) was a much less effective blocker of the calcium influx (data not shown).

DISCUSSION

We have recorded $[Ca^{2+}]_i$ in intact and undissociated rabbit ciliary body epithelial cells. Ciliary body epithelium is a bilayer composed of a non-pigmented cell layer overlying a pigmented one. The cells in the two layers are linked by an extensive network of gap junctions (Raviola & Raviola, 1978). While the $[Ca^{2+}]_i$ measurements were made from non-pigmented cells, the cellular location of the receptors and Ca^{2+}-entry pathways underlying the changes in the $[Ca^{2+}]_i$ are not known. In fact, it is conceivable that some of the changes we have measured originated in the pigmented cells, or that some of the receptors may reside in the membranes of these cells.

We have shown that concurrent activation of muscarinic and α_2-adrenergic receptors in ciliary body epithelium produces a rise in the $[Ca^{2+}]_i$ many times larger than that caused by stimulation of either receptor type alone. Our results (Fig. 1) confirm previous studies on the effects of muscarinic and non-specific adrenergic agonists on $[Ca^{2+}]_i$

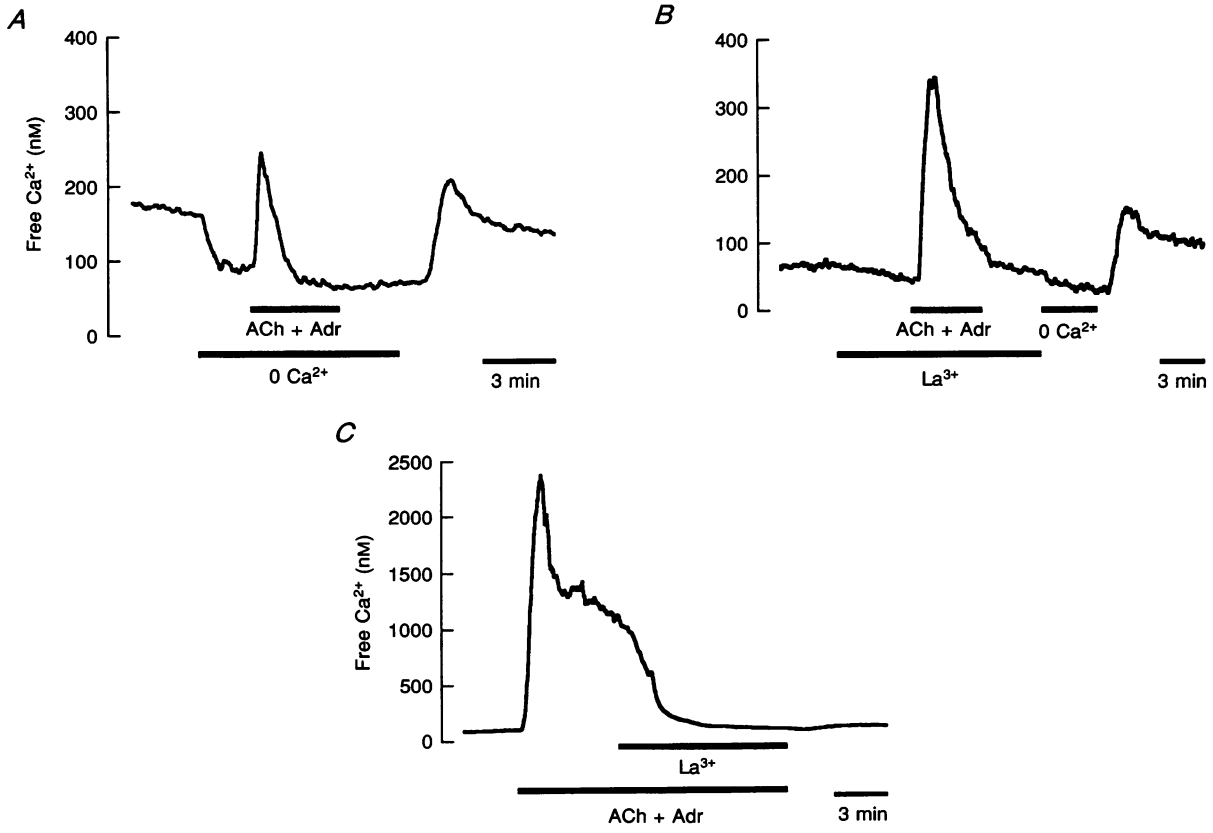

Figure 4. The large increase in $[Ca^{2+}]_i$ caused by ACh and adrenaline is dependent on extracellular Ca^{2+}

A, perfusion of the tissue with low-calcium Ringer solution (0 Ca^{2+}) caused the $[Ca^{2+}]_i$ to decline and significantly reduced the magnitude and duration of the response to ACh and adrenaline (ACh + Adr). Note that the $[Ca^{2+}]_i$ returned to the previous level while the agonists were still present. When extracellular Ca^{2+} was restored to the perfusate, the $[Ca^{2+}]_i$ transiently overshot the original concentration. *B*, a similar response was seen when lanthanum (La^{3+}, 1 μM) was added to the Ringer solution. *C*, in addition, La^{3+} added to the bath in the presence of ACh and adrenaline (ACh + Adr) completely reversed the sustained component of the Ca^{2+} increase.

in this tissue (Lee *et al.* 1989; Ohuchi *et al.* 1992; Crook & Polansky, 1992). For the combined response to ACh and adrenaline, we have shown that the acetylcholine receptor is of the muscarinic type (Fig. 2*A*). Furthermore, we have demonstrated that the adrenergic component is mediated by an α_2-receptor, since it can be activated with UK 14304 and blocked by yohimbine.

There are, however, some indications that the pharmacology of the response to adrenaline is not the same when it is applied alone, in the absence of ACh. Since our data show that adrenaline and noradrenaline increase $[Ca^{2+}]_i$ when applied alone (Fig. 1), but UK 14304 by itself reduced $[Ca^{2+}]_i$ (Fig. 3), this suggests that responses to adrenaline/noradrenaline might be mediated by more than one kind of adrenergic receptor (Mittag & Tormay, 1985). In fact, we have recorded a rise in $[Ca^{2+}]_i$ in response to two other α_2-agonists, clonidine and oxymetazoline, when applied alone (data not shown), though neither of these can fully mimic the synergistic effect of adrenaline when applied with ACh. As suggested by Mittag & Tormay (1985), clonidine (and possibly oxymetazoline) might be activating adrenergic receptors in this tissue with properties intermediate between the α_1- and α_2-types, leading to a rise in $[Ca^{2+}]_i$ similar to the response to stimulation of α_1-receptors in other tissues (Minneman, 1988). Failure of UK 14304 to elicit a rise in $[Ca^{2+}]_i$ in the absence of ACh could be due to rapid desensitization of the α_2-receptor. An alternative explanation might be that the linkage between α_2-receptors and intracellular calcium depends on the presence of an intermediary step, which is provided, in this case, by ACh. The failure of UK 14304 to increase $[Ca^{2+}]_i$ is similar to the finding in human platelets, in which activation of α_2-adrenergic receptors by adrenaline or noradrenaline has no effect on $[Ca^{2+}]_i$, but these same adrenergic agonists potentiate the $[Ca^{2+}]_i$ rise in response to vasopressin, serotonin and platelet-activating factor (Negrescu, Baldenkov, Grigorian, Mazaev & Tkachuk, 1989).

The increase in $[Ca^{2+}]_i$ in response to the combined application of ACh and adrenaline is composed of a transient and sustained component. The transient component might be due to release of Ca^{2+} from intracellular stores (Putney, 1990). However, in the absence of external Ca^{2+} or in the presence of 1 μM La^{3+} only small responses could be recorded (Fig. 4*A* and *B*). This might have been due to depletion of intracellular stores as a result of the drop in $[Ca^{2+}]_i$ under these conditions. Most of the rise in the $[Ca^{2+}]_i$ during the sustained phase of the response is due to Ca^{2+} influx: lowering the extracellular concentration of free Ca^{2+} to 3 nM led to a rapid but reversible fall in $[Ca^{2+}]_i$ (Fig. 1). Furthermore, the elevated level of $[Ca^{2+}]_i$ could be lowered by micromolar concentrations of extracellular La^{3+} (Fig. 4*C*), which has recently been reported to block carbachol-induced Ca^{2+} influx in colonic epithelial cells (Illek, Fischer & Machen, 1992).

The receptor-operated calcium entry pathway in rabbit ciliary body epithelium has not yet been characterized. We have previously shown that this tissue possesses depolarization-activated Ca^{2+} channels similar to L-type channels in neurons (Farahbakhsh, Cilluffo, Chronis & Fain, 1994). However, the sensitivity of the calcium entry pathway activated by ACh/adrenaline to La^{3+} (Fig. 4) and Cd^{2+} is not the same as we have found for the L-type channels (Farahbakhsh *et al.* 1994). In terms of relative sensitivity to La^{3+} and Cd^{2+}, the calcium entry pathway in rabbit ciliary body epithelium resembles the ATP-stimulated calcium influx pathway in rat aortic smooth muscle (Wallnofer, Cauvin, Lategan & Ruegg, 1989). We are currently investigating the effects of dihydropyridines on the ACh/adrenaline-induced $[Ca^{2+}]_i$ increase as well as the effect of ACh and adrenaline on membrane potential to determine if voltage-activated channels have any role in the Ca^{2+} entry.

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