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cGMP abolishes agonist-induced $[Ca^{2+}]_i$ oscillations in human bladder epithelial cells

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Kwan, H. Y., Y. Huang, S. K. Kong, and X. Yao. cGMP abolishes agonist-induced $[Ca^{2+}]_i$ oscillations in human bladder epithelial cells. *Am J Physiol Renal Physiol* 281: F1067–F1074, 2001. First published August 9, 2001; 10.1152/ajprenal.00031.2001.—Cytosolic calcium oscillations may permit cells to respond to information provided by increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) while avoiding prolonged exposure to constantly elevated $[Ca^{2+}]_i$. In this study, we demonstrated that agonists could induce Ca^{2+} oscillations in human bladder epithelial cells. Application of 10 μM acetylcholine or 200 nM bradykinin triggered an initial Ca^{2+} transient that was followed by periodic $[Ca^{2+}]_i$ oscillations. The oscillations did not depend on extracellular Ca^{2+}. 8-Bromoguanosine 3′,5′-cyclic monophosphate abolished acetylcholine- or bradykinin-induced oscillations. Elevation of cellular cGMP by dipyridamole, an inhibitor of cGMP-specific phosphodiesterase, also terminated the $[Ca^{2+}]_i$ oscillations. The inhibitory effect of cGMP could be reversed by KT-5823, a highly specific inhibitor of protein kinase G (PKG), suggesting that the action of cGMP was mediated by PKG. Comparison of the effect of cGMP with that of xestospongin C, an inhibitor of the inositol 1,4,5-trisphosphate (IP_3) receptor, revealed similarities between the action of cGMP and xestospongin C. Therefore, it is likely that cGMP and PKG may target a signal transduction step(s) linked to IP_3 receptor-mediated Ca^{2+} release.

protein kinase G; inositol 1,4,5-triphosphate; calcium release; nitric oxide; intracellular calcium concentration; guanosine 3′,5′-cyclic monophosphate

OSCILLATORY CHANGES IN INTRACELLULAR Ca^{2+} concentration ($[Ca^{2+}]_i$), or $[Ca^{2+}]_i$ oscillations, occur in a variety of nonexcitable cell types (10, 14, 18, 34). $[Ca^{2+}]_i$ oscillations can be triggered by a great variety of stimuli, including neurotransmitters, hormones, growth factors, and mechanical stress. Of the natural stimuli, many are calcium-mobilizing agents that bind to cell surface receptors and then activate phospholipase C. This leads to the breakdown of phosphatidylinositol 4,5-biphosphate into two important second messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (4). IP_3 binds to its receptor, which acts as a Ca^{2+} channel in the endoplasmic reticular membrane and triggers the releases of Ca^{2+} into cytoplasm (11).

A number of mechanistic models for $[Ca^{2+}]_i$ oscillations have been proposed (9, 25). The single-pool model hypothesizes that IP_3 activates its receptor and releases Ca^{2+} from a single intracellular Ca^{2+} pool. The resulting elevation in cytosolic Ca^{2+} then feeds back to inhibit further release of Ca^{2+} by the IP_3 receptor (28). Rapid activation of the IP_3 receptor by IP_3 and slow inactivation of the IP_3 receptor by Ca^{2+} as $[Ca^{2+}]_i$ increases to higher values, along with the functioning of Ca^{2+}-ATPase at the endoplasmic reticular membrane, lead to cytosolic Ca^{2+} oscillations (9, 24, 25). An alternative two-pool model suggests that two separate intracellular Ca^{2+} stores may be involved in the generation of cytosolic Ca^{2+} oscillations, one being an IP_3-sensitive store and the other an IP_3-insensitive one (3).

cGMP has distinct effects on intracellular Ca^{2+} levels in different cells, decreasing free $[Ca^{2+}]_i$ in smooth muscles (26), cardiac myocytes (28), platelets (33), and megakaryocytes (39) and increasing $[Ca^{2+}]_i$ in hepatocytes (34) and sea urchin eggs (13). Multiple targets for cGMP have been identified. cGMP inhibits IP_3 formation in smooth muscle cells (26), inhibits Ca^{2+} entry into smooth muscle and endothelial cells (26, 43), and inhibits IP_3 receptor-mediated Ca^{2+} release from endoplasmic reticulum in smooth muscle cells and megakaryocytes (26, 39). There are only a few studies on the effect of cGMP on $[Ca^{2+}]_i$ oscillations. In rat megakaryocytes, application of cGMP inhibits ATP-induced $[Ca^{2+}]_i$ oscillations (38, 39). In contrast, cGMP is reported to initiate $[Ca^{2+}]_i$ oscillations via stimulating IP_3 receptor-mediated Ca^{2+} release in rat hepatocytes (34).

In the present study, we used laser scanning confocal microscopy to measure acetylcholine- or bradykinin-induced $[Ca^{2+}]_i$ oscillations in human bladder epithelial cells. We found that both acetylcholine- and bradykinin-induced cytoplasmic Ca^{2+} oscillations in human bladder epithelial cells could be inhibited by cGMP via a protein kinase G (PKG)-dependent mechanism.

MATERIALS AND METHODS

Materials. Fluo 3-acetoxymethyl ester (AM) and Pluronic F127 were obtained from Molecular Probes. Tissue culture

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media and materials were from GIBCO BRL. Culture flasks and culture plates were from Becton Dickinson. Acetylcholine, bradykinin, atropine, carbachol, 8-bromoadenosine 3′,5′-cyclic monophosphate (8-BrcAMP), 8-BrcGMP, KT-5823, xestospongin C (XeC), cyclopiazonic acid (CPA), and dipyridamole were from Calbiochem. HEPES and EDTA were purchased from Sigma. HOE-40 was from RBI.

Cell culture. ECV304 is a human bladder epithelial cell line identical to T24/83 (6). Cells were cultured in 90% RPMI-1640 and 10% fetal bovine serum (FBS) containing 100 U/ml penicillin and 100 μg/ml streptomycin and incubated in T-75 tissue culture flasks at 37°C in an atmosphere of 5% CO_2-95% air. Confluent cell monolayers were passaged using 0.25% trypsin containing 2.5 mM EDTA.

$[Ca^{2+}]_i$ measurement. ECV304 cells were grown overnight in 90% RPMI-1640 supplemented with 10% FBS containing 100 U/ml penicillin and 100 μg/ml streptomycin on circular disks (Fisher 25 CIR-1) at 37°C and 5% CO_2-95% air. Cells were loaded with fluo 3-AM for 1 h in the dark at room temperature by incubation with 10 μM membrane-permeant fluo 3-AM and 0.02% Pluronic F127 in a normal physiological saline solution (N-PSS) containing (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, and 5 HEPES (pH 7.4). After loading of fluo 3-AM, cells were transferred to Ca^{2+}-free PSS to remove excessive external fluo 3-AM. The circular disks containing the ECV304 epithelial cells were then pinned in a specially designed chamber. The chamber was placed on the stage of an inverted microscope (Nikon Diaphot 200), and the fluorescence signal was recorded by the MRC-1000 laser scanning confocal imaging system with MRC-1000 software (Bio-Rad). Experiments were performed without flow. Cells were bathed in Ca^{2+}-free PSS that contained (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 0.2 EGTA, 10 glucose, and 5 HEPES (pH 7.4). All agents were applied directly to the bath at the side of the chamber; solutions were then mixed by gentle pipetting. As a control, pipetting with bath media in the absence of agonists did not produce any change in $[Ca^{2+}]_i$. Data analysis was performed with the Confocal Assistant and Metafluor systems (Bio-Rad). Changes in $[Ca^{2+}]_i$ in response to all agents were displayed as the ratio of fluorescence relative to the fluorescence before the application of agents (F_0).

RESULTS

Responses to acetylcholine and bradykinin. The Ca^{2+} responses to acetylcholine and bradykinin in cultured human bladder epithelial cells loaded with the Ca^{2+} indicator fluo 3 were monitored by laser scanning confocal microscopy. The application of 10 μM acetylcholine greatly changed the intracellular Ca^{2+} levels. Three basic responses to acetylcholine were observed. These consisted of *1*) a single Ca^{2+} transient after exposure to ATP (Fig. 1*D*); *2*) initiation of $[Ca^{2+}]_i$ oscillations (Fig. 1, *A* and *B*); and *3*) a prolonged elevation

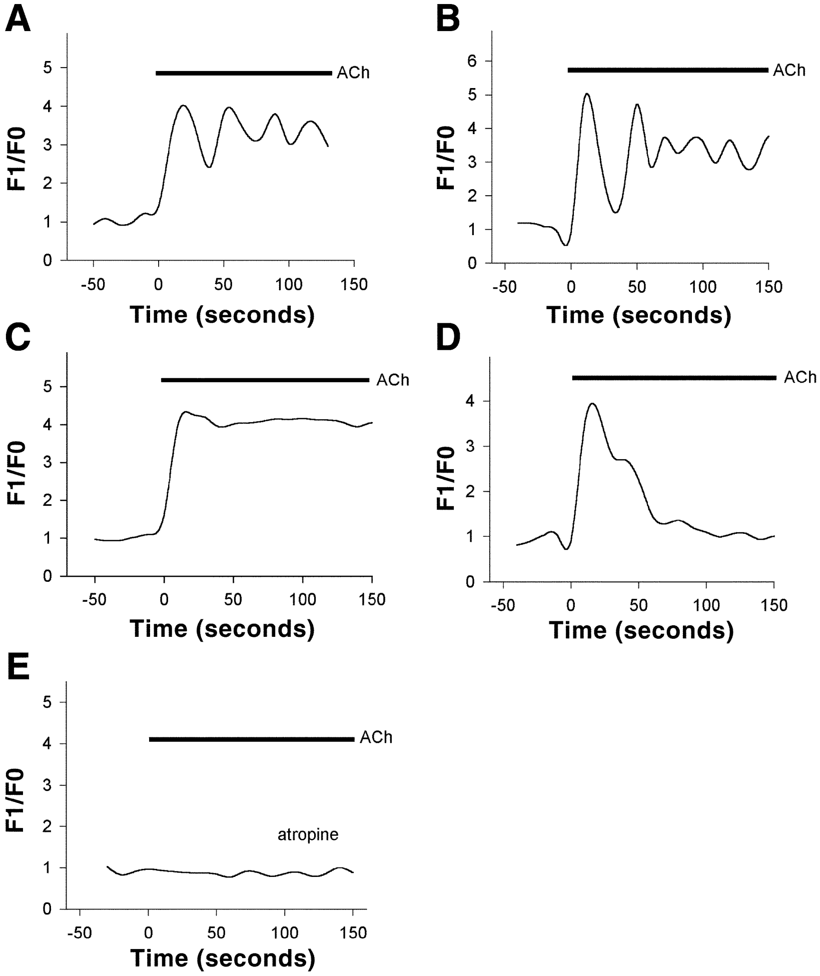

Fig. 1. ACh-induced intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) oscillations in bladder epithelial cells. *A–D*: variation in $[Ca^{2+}]_i$ responses to ACh among different cells. Each trace represents a separate cell. Cells were grown thinly and placed in a Ca^{2+}-free physiological saline solution (0Ca-PSS). ACh (10 μM) was added. *E*: effect of atropine (100 nM) on ACh-induced $[Ca^{2+}]_i$ oscillations. F_1/F_0, relative fluorescence. Curves are typical of the data obtained from 4–5 experiments, comprising a total of 60–80 cells.

of baseline $[Ca^{2+}]_i$ (Fig. 1*C*). Acetylcholine (10 μM) induced an initial Ca^{2+} transient in ~85% of cells. After the initial Ca^{2+} increase induced by acetylcholine, 35–65% of cells, depending on the preparations, displayed periodic $[Ca^{2+}]_i$ oscillations. The frequency of oscillations varied from 1 to 2 Hz, whereas the amplitude of oscillations varied greatly and, in general, decreased with time. The mean peak amplitude (F_1/F_0) of the first $[Ca^{2+}]_i$ oscillation was calculated to be 3.8 ± 0.2 (*n* = 10). For comparison, the peak amplitude of the fifth oscillation was ~30% lower at 2.7 ± 0.2 (*n* = 10). The oscillations did not depend on Ca^{2+} influx because the oscillatory activity could be recorded in cells bathed in Ca^{2+}-free PSS. Acetylcholine washout immediately halted the oscillatory responses. The acetylcholine-induced $[Ca^{2+}]_i$ oscillations were not related to the hydrolysis of acetylcholine because 50 μM carbachol was also able to induce the oscillations. Incubation of cells for 5 min in atropine (100 nM), a muscarinic receptor antagonist, completely abolished the initial Ca^{2+} transient as well as the subsequent Ca^{2+} oscillations (Fig. 1*E*, *n* = 3).

Similarly, bradykinin was also able to induce $[Ca^{2+}]_i$ oscillations. Application of 200 nM bradykinin triggered an initial Ca^{2+} transient in >95% of cells, and this was followed by periodic $[Ca^{2+}]_i$ oscillations in ~80% of cells (Fig. 2, *A–D*). The oscillatory activity elicited by bradykinin ceased immediately on bradykinin washout. Incubation of cells for 5 min in HOE-140 (1 μM), a selective B_2 bradykinin receptor antagonist, completely abolished bradykinin-induced initial Ca^{2+} transient as well as the subsequent $[Ca^{2+}]_i$ oscillations (Fig. 2*E*, *n* = 6).

In agreement with what has been observed in rabbit airway epithelial cells (10) and rat pituitary gonadotrophs (25), the oscillatory responses of $[Ca^{2+}]_i$ in human bladder epithelial cells were also greatly affected by the agonist concentration applied. As the concentration of applied ATP increased from low to intermediate to high, the response of cells shifted from a single Ca^{2+} transient to $[Ca^{2+}]_i$ oscillations and then to prolonged $[Ca^{2+}]_i$ elevation (Table 1). In addition, the peak amplitudes of Ca^{2+} responses also increased with increasing ATP concentrations (Table 1). Similar dose-dependent differential $[Ca^{2+}]_i$ responses could also be observed for bradykinin at concentrations between 10 and 200 nM (data not shown). We also attempted to test whether individual cells within a population exhibited differing sensitivities to agonists by applying increasing concentrations of agonists in a stepwise

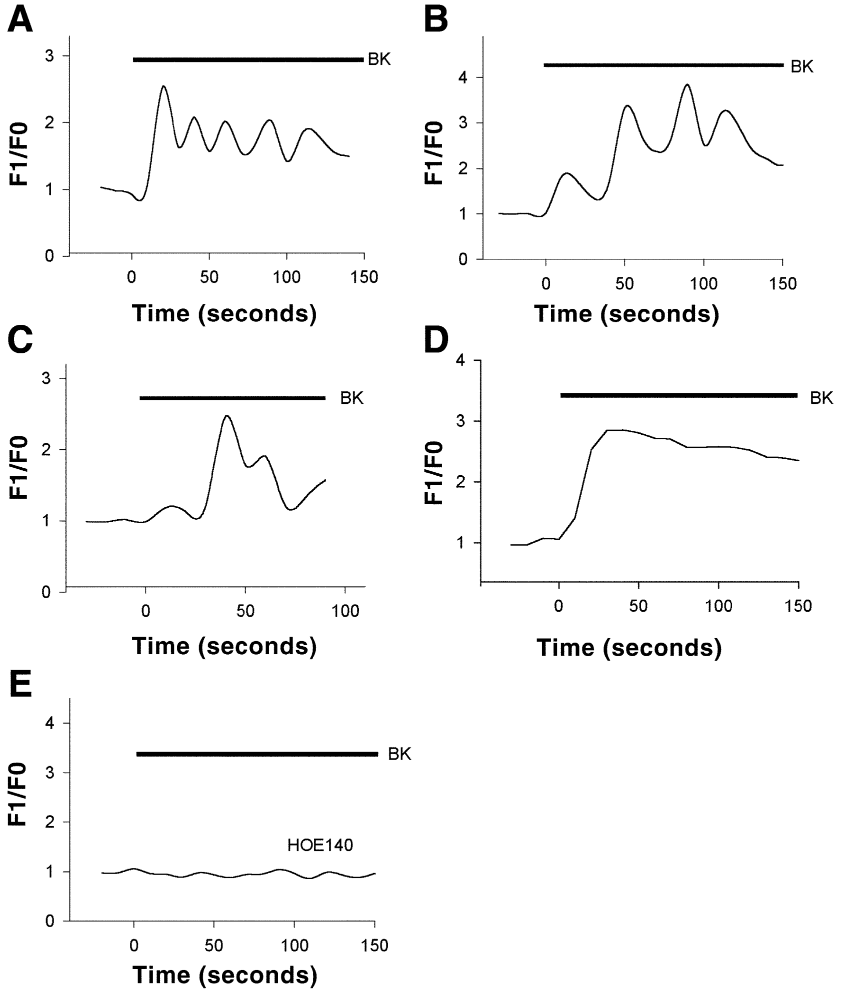

Fig. 2. Bradykinin (BK)-induced $[Ca^{2+}]_i$ oscillations in bladder epithelial cells. *A–D*: variation in $[Ca^{2+}]_i$ responses to BK among different cells. Each trace represents a separate cell. Cells were grown thinly and placed in 0Ca-PSS. BK (200 nM) was added. *E*: effect of HOE-140 (1 μM) on BK-induced $[Ca^{2+}]_i$ oscillations. Curves are typical of the data obtained from 6–10 experiments, comprising a total of 90–150 cells.

Table 1. *Effect of acetylcholine concentration on $[Ca^{2+}]_i$ responsiveness in cultured human bladder epithelial cells*

ACh Concentration, μM	Percentage of Cells Displaying Different $[Ca^{2+}]_i$ Responses				Peak F_1/F_0
	No response	Single transient	Oscillation	Plateau	
0.1	94 ± 3% (3)	6 ± 3% (3)	0 ± 0% (3)	0 ± 0% (3)	1.4 ± 0.2 (3)
1	21 ± 3% (4)	43 ± 9% (4)	30 ± 7% (4)	6 ± 4% (4)	2.3 ± 0.2 (4)
10	9 ± 5% (5)	6 ± 2% (5)	55 ± 10% (5)	30 ± 14% (5)	3.8 ± 0.2 (5)
20	0 ± 0% (3)	0 ± 0% (3)	2 ± 1% (3)	98 ± 2% (3)	4.2 ± 0.3 (3)

Values are means ± SE from 3–5 experiments, containing a total of 30–75 cells, and are expressed as a percentage, with no. of experiments in parentheses. $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; F_1/F_0, mean peak amplitude.

manner to a single dish of cells. However, we found that treatment of cells with ATP or bradykinin, even at low concentrations, reduced and sometimes abolished the Ca^{2+} responses to subsequent agonist challenge. In other words, the Ca^{2+} responses of individual cells desensitized after their preexposure to agonists. For this reason, our protocol failed to resolve whether individual cells within an exhibited population had differing sensitivities to agonists.

One previous report suggested that extracellular EGTA might interfere with histamine-induced Ca^{2+} release from intercellular Ca^{2+} stores in airway epithelial cells (15). In our experiments, however, no apparent differences in oscillatory responses could be found between the cells bathed in Ca^{2+}-free PSS that contained 0.2 mM EGTA and a nominally Ca^{2+}-free solution containing no EGTA, suggesting that EGTA at the concentration used did not influence the oscillatory activity elicited by agonists. Changes in cell culture media from RPMI-1640 to DMEM or α-MEM did not affect the agonist-induced oscillatory responses either.

Besides calcium-mobilizing agonists, flow shear stress is another factor that may initiate Ca^{2+} oscillations, at least in cultured vascular endothelial cells (16, 36). We therefore tested the effect of mechanical stimulation generated by flow on Ca^{2+} signaling in cultured bladder epithelial cells. In this series of experiments, flow was initiated by pumping N-PSS or Ca^{2+}-free PSS to a specially designed flow chamber so that appropriate shear force could be generated. Shear force at the range of 0.1–10 dyn/cm^2 elicited a transient Ca^{2+} elevation, but without Ca^{2+} oscillations, in cells perfused by Ca^{2+}-containing N-PSS. The flow-induced Ca^{2+} transient was dependent on extracellular Ca^{2+}, because perfusion by Ca^{2+}-free PSS could not elicit the Ca^{2+} transient. Therefore, it appears that flow and agonists may stimulate cytosolic Ca^{2+} changes through different mechanisms in bladder epithelial cells.

Effect of cGMP and KT-5823 on $[Ca^{2+}]_i$ oscillations. cGMP is an intracellular second messenger that activates PKG. We used 8-BrcGMP, a membrane-permeant cGMP analog, to activate PKG and a highly specific inhibitor, KT-5823, to inhibit PKG. Addition of 2 mM 8-BrcGMP immediately stopped ongoing acetylcholine- or bradykinin-induced $[Ca^{2+}]_i$ oscillations (Fig. 3, *A* and *B*). Application of 1 μM KT-5823 reversed the effect of cGMP and reinitiated the transient rise of intracellular Ca^{2+}. In ~50% of cells, this initial Ca^{2+} transient was followed by $[Ca^{2+}]_i$ oscillations (Fig. 3). As a control, KT-5823 alone was not able to initiate $[Ca^{2+}]_i$ oscillations ($n = 3$). In separate experiments, cells were preincubated in 2 mM 8-BrcGMP for 5 min; the preincubation completely abolished the acetylcholine- or bradykinin-induced initial Ca^{2+} transient as well as the subsequent $[Ca^{2+}]_i$ oscillations ($n = 5$). If cGMP preincubation was carried out in the presence of 1 μM KT-5823, cGMP was not able to inhibit acetylcholine- or bradykinin-induced $[Ca^{2+}]_i$ oscillations (Fig. 4, *A* and *B*).

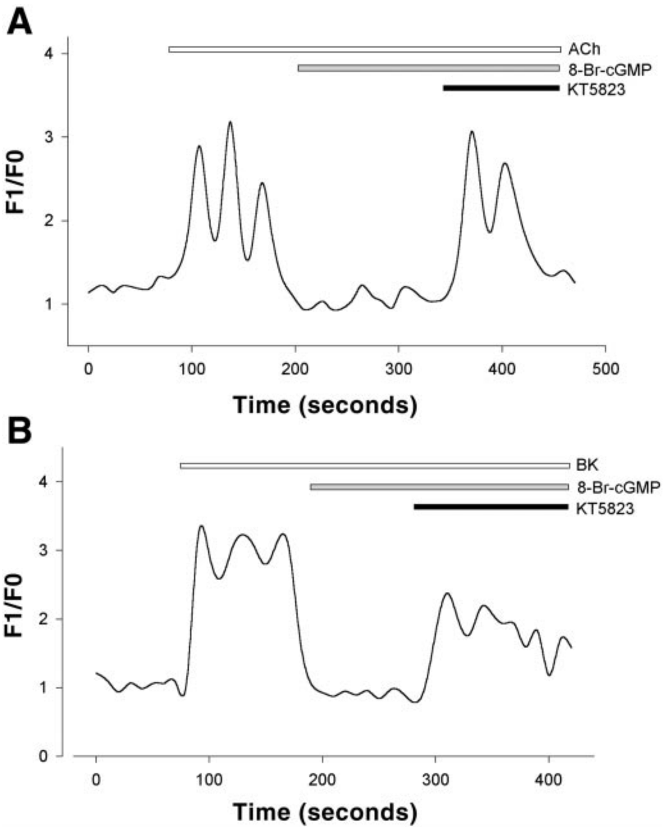

Fig. 3. Effect of 8-bromoguanosine 3′,5′-cyclic monophosphate (8-BrcGMP) and KT-5823 on ACh- or BK-induced $[Ca^{2+}]_i$ oscillations. 8-BrcGMP inhibited ACh (*A*)- or BK-induced (*B*) $[Ca^{2+}]_i$ oscillations. KT-5823 reversed the inhibition. Cells were grown thinly and placed in 0Ca-PSS. Chemicals were added sequentially as follows: 10 μM ACh; 200 nM BK; 2 mM 8-BrcGMP; and 1 μM KT-5823. Each curve is typical of the data obtained from 3–9 experiments, comprising a total of 50–150 cells.

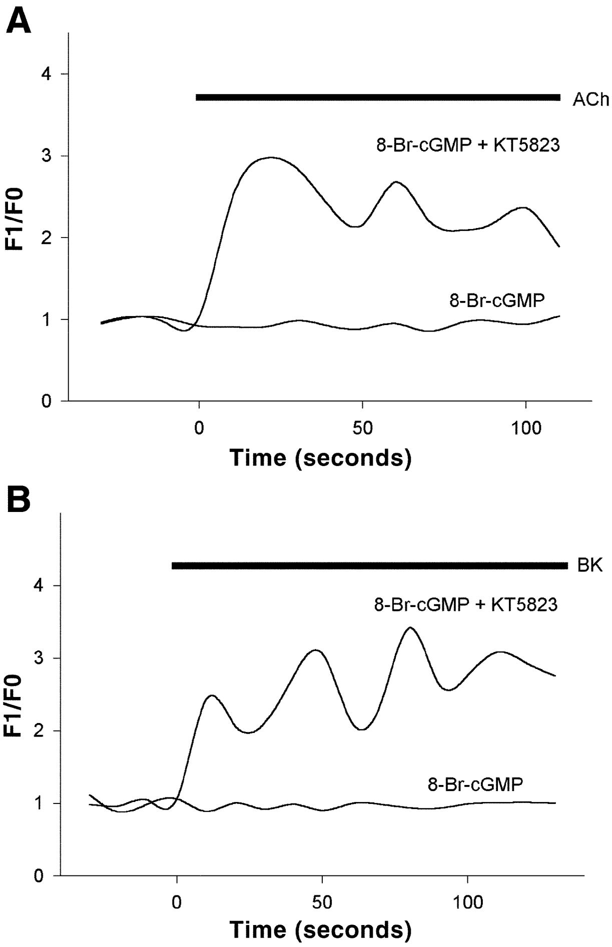

Fig. 4. Effect of preincubation in 8-BrcGMP and KT-5823 on $[Ca^{2+}]_i$ oscillations. Preincubation of cells in 8-BrcGMP abolished ACh (*A*)- or BK-induced (*B*) $[Ca^{2+}]_i$ oscillations. KT-5823 reversed the inhibition. Cells were grown thinly and placed in 0Ca-PSS. Chemicals were added as shown: 10 μM ACh; 200 nM BK; 2 mM 8-BrcGMP; and 1 μM KT-5823. Each curve is typical of the data obtained from 3–7 experiments, comprising a total of 50–110 cells.

It appeared that KT-5823 treatment also increased the percentage of cells responding to acetylcholine. After KT-5823 treatment, the percentage of cells demonstrating the acetylcholine-induced $[Ca^{2+}]_i$ oscillations was 98 ± 4% ($n = 5$). Without the treatment, only 30 ± 3% ($n = 5$) cells displayed the acetylcholine-induced $[Ca^{2+}]_i$ oscillations. On the other hand, the percentage of cells demonstrating bradykinin-induced $[Ca^{2+}]_i$ oscillations did not change significantly after KT-5823 treatment (before treatment: 82 ± 7%, $n = 10$; after treatment: 90 ± 6%, $n = 4$).

Taken together, our results suggest that acetylcholine- or bradykinin-induced $[Ca^{2+}]_i$ oscillations in human bladder epithelial cells are regulated by a PKG-dependent mechanism. Activation of PKG by 8-BrcGMP abolishes $[Ca^{2+}]_i$ oscillations, whereas the inhibition of PKG by KT-5823 reinstates them.

It has been reported that PKG and PKA have similarities in structure and substrate specificity (2). We therefore tested the effect of 8-BrcAMP on acetylcholine- or bradykinin-induced Ca^{2+} oscillations. Unlike with cGMP, preincubation of cells with 2 mM 8-BrcAMP had no effect on the acetylcholine- or bradykinin-induced $[Ca^{2+}]_i$ oscillations, suggesting that PKA was not involved ($n = 3$).

Effect of dipyridamole on $[Ca^{2+}]_i$ oscillations. Dipyridamole is an inhibitor of cGMP-specific phosphodiesterase V. It raises intracellular cGMP levels by inhibiting cGMP degradation via phosphodiesterase (41). Incubation of cells for 5 min in 10 μM dipyridamole before the application of 10 μM acetylcholine (Fig. 5*A*) or 200 nM bradykinin (Fig. 5*B*) completely abolished the agonist-induced initial Ca^{2+} transient as well as the subsequent $[Ca^{2+}]_i$ oscillations. These data are consistent with the inhibitory role of cGMP in agonist-induced $[Ca^{2+}]_i$ oscillations.

Effect of CPA and XeC on $[Ca^{2+}]_i$ oscillations. To define the importance of the IP_3 receptor in acetylcholine-induced $[Ca^{2+}]_i$ oscillations, we used a membrane-permeable blocker, XeC, to selectively inhibit the receptor (12). Application of 5 μM XeC immediately ceased ongoing acetylcholine- or bradykinin-induced

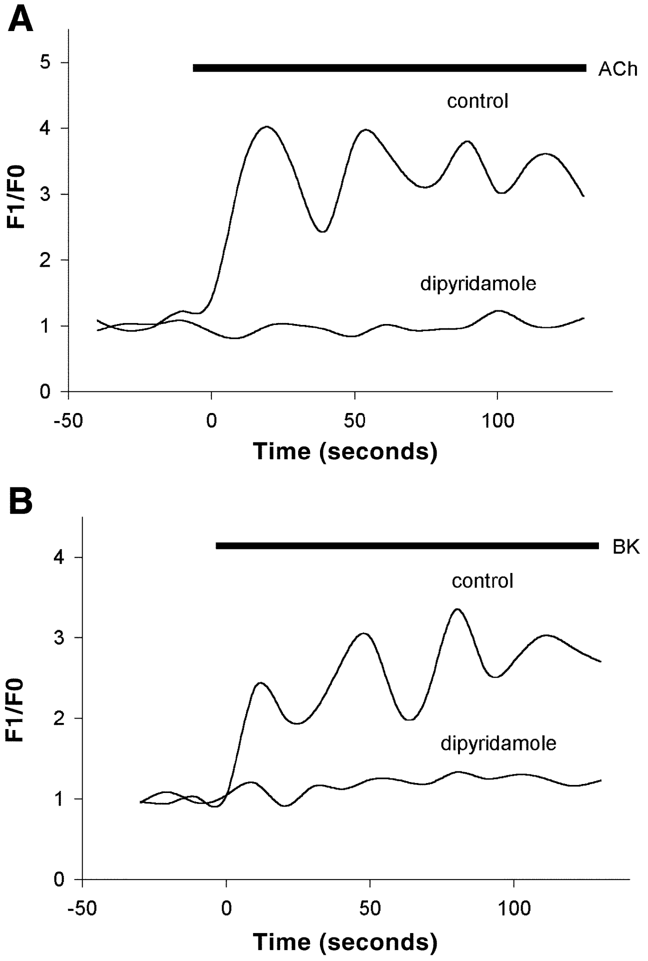

Fig. 5. Effect of dipyridamole on ACh- or BK-induced $[Ca^{2+}]_i$ oscillations. Incubation of cells in dipyridamole abolished ACh (*A*)- or BK-induced (*B*) $[Ca^{2+}]_i$ oscillations. Cells were grown thinly and placed in 0Ca-PSS. Chemicals were added as shown: 10 μM ACh, 200 nM BK, and 10 μM dipyridamole. Each curve is typical of the data obtained from 3 experiments, comprising a total of ~50 cells.

$[Ca^{2+}]_i$ oscillations (Fig. 6*A*). In separate experiments, when cells were pretreated with 5 μM XeC, acetylcholine or bradykinin failed to induce the initial Ca^{2+} transient as well as the subsequent $[Ca^{2+}]_i$ oscillations (Fig. 6*B*). These data suggest that IP_3 receptor-mediated Ca^{2+} release is required for $[Ca^{2+}]_i$ oscillations.

The role of sarcoplasmic or endoplasmic reticular Ca^{2+}-ATPase (SERCA) in $[Ca^{2+}]_i$ oscillations was examined with the use of CPA, a selective inhibitor of SERCA (30). Preincubation of cells for 1 min in 10 μM CPA had no effect on the rising phase of the initial Ca^{2+} signal elicited by acetylcholine. In the presence of CPA, however, $[Ca^{2+}]_i$ remained at an elevated level over the time course of experiments after it had reached its peak (Fig. 7*A*). These results suggest that the falling phase of the initial Ca^{2+} transient is mostly due to the activity of SERCA.

The acetylcholine-induced $[Ca^{2+}]_i$ rise in the presence of CPA was also subjected to the regulation by cGMP and PKG. Incubation of cells for 5 min in 2 mM 8-BrcGMP abolished the acetylcholine-induced $[Ca^{2+}]_i$ rise. In the presence of 1 μM KT-5823, cGMP had no effect (Fig. 7*B*).

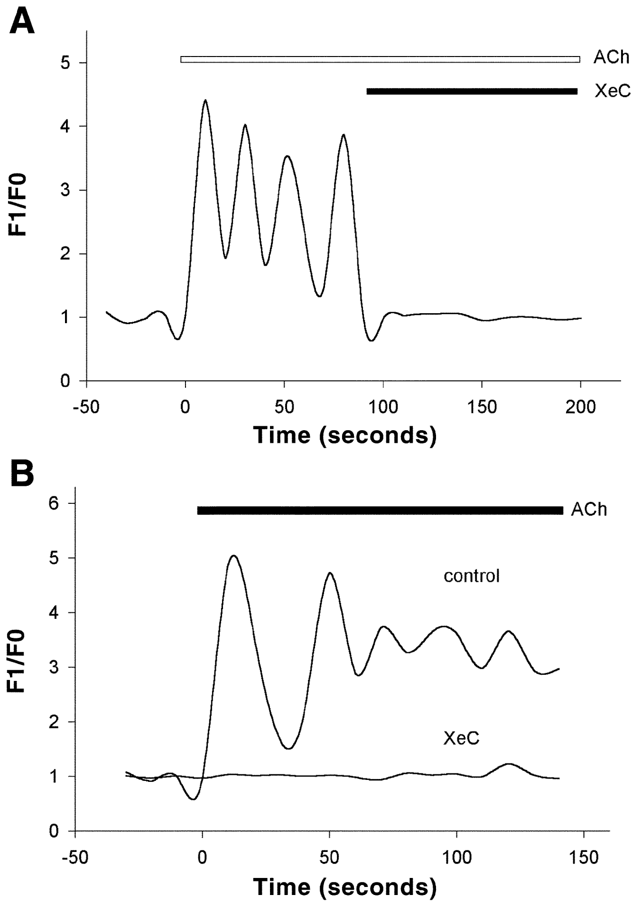

Fig. 6. Effect of xestospongin C (XeC) on ACh-induced $[Ca^{2+}]_i$ oscillations. XeC abolished ACh-induced $[Ca^{2+}]_i$ oscillations. Cells were grown thinly and placed in 0Ca-PSS. Chemicals were added as shown: 10 μM ACh and 5 μM XeC. Each curve is typical of the data obtained from 3–4 experiments, comprising a total of 40–60 cells.

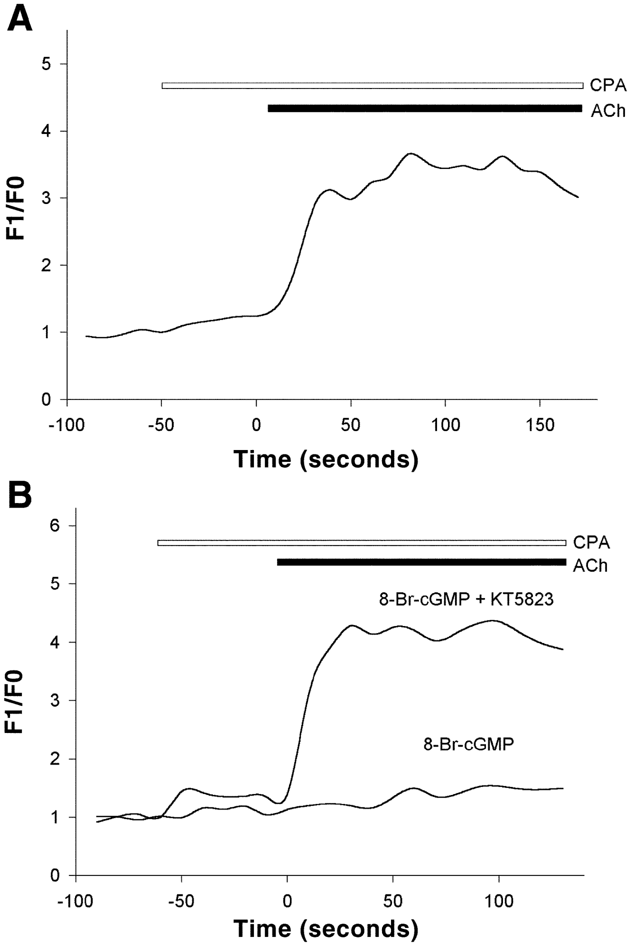

Fig. 7. Effect of cyclopiazonic acid (CPA) on ACh-induced Ca^{2+} transient. *A*: CPA diminished the falling phase of the ACh-induced Ca^{2+} transient. *B*: 8-BrcGMP abolished the ACh-induced Ca^{2+} transient, and KT-5823 reversed the inhibition. Cells were grown thinly and placed in 0Ca-PSS. Chemicals were added as shown: 10 μM ACh; 10 μM CPA; 2 mM 8-BrcGMP; and 1 μM KT-5823. Each curve is typical of the data obtained from 3–4 experiments, comprising a total of 40–60 cells.

DISCUSSION

Calcium signaling in nonexcitable cells regulates such diverse processes as gene regulation, secretion, apoptosis, and cell proliferation. In bladder epithelial cells, intracellular calcium is known to regulate Na^+ reabsorption and proton secretion (23, 35), alter antidiuretic hormone-mediated osmotic water flow (7), participate in cell volume regulation (42), control granule exocytosis (29), and regulate the insertion of H^+-ATPase into the apical membrane (40). Ca^{2+}-sensitive cell functions are often mediated by oscillatory rather than prolonged sustained increases in $[Ca^{2+}]_i$ (32). The advantages of these oscillatory signals include *1*) favorable signal-to-noise ratios (32) and *2*) avoidance of the adverse effects of sustained elevation in $[Ca^{2+}]_i$ (27). Oscillatory $[Ca^{2+}]_i$ signals can be decoded into changes in Ca^{2+}/calmodulin-dependent protein kinase II activity (22) and nuclear factor-kB transcriptional activity (17). Until now, however, there was still a lack of evidence for $[Ca^{2+}]_i$ oscillations in bladder epithelial cells. In the present study, we demonstrated the existence of agonist-induced $[Ca^{2+}]_i$ oscillations in human bladder epithelial cells. The oscillations elicited by acetylcholine were inhibited by atropine, suggesting that the action of acetylcholine was mediated by muscarinic receptors. The oscillations elicited by bradykinin could be abolished by HOE-140, implicating the involvement of the B_2 bradykinin receptor. As in many other cell types (10, 34), agonist-induced Ca^{2+} oscillations in bladder epithelial cells did require the presence of extracellular Ca^{2+}.

The effect of cGMP on $[Ca^{2+}]_i$ oscillations appears to depend on the cell type. cGMP stimulates $[Ca^{2+}]_i$ oscillations in rat hepatocytes, whereas it inhibits $[Ca^{2+}]_i$ oscillations in rat megakaryocytes (34, 38, 39). The effect of cGMP could be caused by the direct action of cGMP (19), be mediated by a G kinase (26), result from the activation of PKA (2), or be due to increases in cAMP that result from an inhibition of cAMP phosphodiesterase activity (1). In our experiments, application of cGMP abolished the acetylcholine- or bradykinin-induced $[Ca^{2+}]_i$ oscillations in cultured human bladder epithelial cells (Figs. 3 and 4). The inhibitory effect was reversed by a highly specific PKG inhibitor, KT-5823 (Figs. 3 and 4). Membrane-permeant 8-BrcAMP had no effect on $[Ca^{2+}]_i$ oscillations. These data suggest that the effect of cGMP is mediated by PKG and argue against either a direct effect of cGMP or an indirect effect due to an increase in cAMP or activation of PKA.

The $[Ca^{2+}]_i$ oscillations in human bladder epithelial cells require the functioning of both the IP_3 receptor and SERCA. The rising phase of $[Ca^{2+}]_i$ oscillations may result from Ca^{2+} release through the IP_3 receptor, whereas the falling phase of the oscillations may be attributable to Ca^{2+} sequestration into intracellular stores as well as Ca^{2+} extrusion into the extracellular medium (24). In our experiments, a membrane-permeant IP_3 receptor inhibitor, XeC, abolished the acetylcholine- or bradykinin-induced $[Ca^{2+}]_i$ oscillations (Fig. 6). Application of CPA, which blocks SERCA and results in the inability of the sarcoplasmic reticulum to sequester Ca^{2+}, diminished the falling phase of Ca^{2+} signals (Fig. 7*A*). These data suggest that the Ca^{2+} sequestration into intracellular Ca^{2+} stores is the main mechanism responsible for the falling phase of agonist-induced $[Ca^{2+}]_i$ transients and/or oscillations, whereas Ca^{2+} extrusion to the extracellular medium may only play a minor role.

In our experiments, action of cGMP on intracellular Ca^{2+} was similar to that of XeC but was apparently different from that of CPA. Preincubation of cells in cGMP or XeC completely abolished the agonist-induced initial Ca^{2+} transient as well as the subsequent $[Ca^{2+}]_i$ oscillations. In contrast, CPA did not influence the rising phase of the initial Ca^{2+} transient. Furthermore, in the presence of CPA, cGMP could still abolish the acetylcholine-induced Ca^{2+} transient (Fig. 7*B*). These results suggest that cGMP and PKG may act like XeC and target some signaling step(s) linked to the IP_3 receptor-mediated Ca^{2+} release. The present data cannot distinguish the precise step in which PKG may act. cGMP may inhibit IP_3 formation, as in smooth muscle cells, or it may directly inhibit the IP_3 receptor, as in smooth muscle cells and megakaryocytes (26, 39).

Nitric oxide (NO) is produced in urinary bladder epithelial cells (5, 8) and in the nerves supplying the bladder (20). It can cause smooth muscle relaxation in the lower urinary tract (20). Changes in the NO level in bladder epithelium have been implicated in the pathogenesis of bladder tumors (21). As in many other cell types (26), the action of NO in uroepithelial cells is likely to be mediated by cGMP (37). NO may activate guanylate cyclase, leading to the elevation of cGMP levels in bladder epithelial cells (37, 44). Our present data provide a possible target for this NO-cGMP signaling pathway in bladder epithelium. It is possible that NO-cGMP may target the $[Ca^{2+}]_i$ oscillations in bladder epithelium. The change in Ca^{2+} oscillations may then regulate other processes, such as gene transcription and cell proliferation.

In conclusion, we find that cultured human bladder epithelial cells display acetylcholine- and bradykinin-induced $[Ca^{2+}]_i$ oscillations. The oscillatory activity requires the functioning of the IP_3 receptor as well as SERCA. cGMP, via its action on PKG, may affect the signaling pathway, leading to IP_3 receptor-mediated Ca^{2+} release, thus regulating $[Ca^{2+}]_i$ oscillations.

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