Intracellular Ca^{2+} regulation and electrophysiolgical properties of bladder urothelium subjected to stretch and exogenous agonists

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Abstract

Intracellular Ca^{2+} control and the electrophysiological properties of guinea-pig urothelium were measured during interventions encountered during bladder filling, including cell stretch and exposure to exogenous transmitters such as ATP and muscarinic agonists. Stretch, achieved by exposure to solutions of altered osmolality, generated intracellular Ca^{2+}-transients that were attenuated by Gd^{3+} in isolated cells. However ATP-induced intracellular Ca^{2+}-transients were unaffected by Gd^{3+} but blocked by thapsigargin. ATP-dependent Ca^{2+}-transients were followed by a large inward current at a holding potential of -60 mV. Carbachol was without significant effect, except for a small slowing of the rate of spontaneous intracellular Ca^{2+}-transients that were recorded in about one-third of cells. With urothelial sheets the transepithelial potential (TEP) was increased by ATP applied to the baso-lateral (serosal) face, a similar change was achieved by reduction of the basolateral [Na]; carbachol was without significant effect. We propose that a rise of intracellular Ca^{2+} may control ATP release as both mechanical stretch and exogenous ATP have been shown previously to release further ATP from isolated urothelium as part of a postulated signalling pathway for bladder filling. The similar increase of TEP by ATP and a raised transepithelial Na gradient is also consistent with a role for transepithelial ion transport as a regulator of ATP release. The lack of large effects with carbachol implies muscarinic agonists must exert any effects on the urothelium through other pathways.

Introduction

Stretch of the urothelium that lines the bladder wall, as occurs during bladder filling, releases ATP and acetylcholine from the baso-lateral (serosal) surface and is hypothesised as the initial stage in sensations of bladder filling [1,2]. There is increased ATP release in tissue from bladders with pathologies associated with overactivity [3], which is normalised in such bladders treated with botulinum toxin [4]. ATP may even exert a positive feedback control over its own release, demonstrated in cultured urothelial cells [3]. Moreover, it is widely accepted that antimuscarinic agents act during the filling, rather than the emptying, phase of the micturition cycle [5], but it is not known if muscarinic agonists modulate this transmitter release or other physiological properties of the urothelium.

A more detailed characterisation of transmitter release is hampered by a lack of knowledge of the intracellular pathways in urothelial cells that may mediate such release and how the transmitters themselves exert autocrine control. A range of receptors to potential transmitters, including those to purinergic and muscarinic ligands, have been labelled on urothelial cells [6-8], but their functional significance has not been identified. In particular, it remains controversial if transmitter release is dependent on changes to the intracellular [Ca^{2+}] [9,10] and it is also unclear if any of the potential stimulators of release can elicit such changes.

The aim of this study was to characterise the actions of interventions that physically stress urothelial cells as well as urothelial transmitters on the urothelium itself. In particular their ability to alter intracellular Ca^{2+} signalling and the electrophysiological properties of the urothelium were investigated, to gain insight into their potential mode of action.

Methods

Animals and experimental preparations. Guinea-pigs (Dunkin-Hartley, 350-500 g) were killed by cervical dislocation in compliance with the UK Animals (Scientific Procedures) Act 1986, the urinary bladder removed and placed in a nominally Ca^{2+}-free solution. The mucosa (urothelium and suburothelium) was separated from the detrusor layer by blunt dissection, minimising touch of the mucosa. The mucosa sheets were used either for Ussing chamber experiments (below) or dissociated into isolated cells for intracellular Ca^{2+} recording [11]. Briefly, the urothelium sheet was incubated in an enzyme-containing HEPES-buffered Ca^{2+}-free solution at room temperature for 10 minutes, cut into small pieces and then gently stirred at 37°C for a further 10 minutes. After incubation the enzyme solution was discarded by centrifugation (700g) and the cell pellet resuspended in HEPES-buffered Ca^{2+}-free solution and stored at 4°C for use on the same day. A drop of cell-containing solution was placed in a superfusion chamber on the stage of an inverted microscope. Spherical cells were chosen rather than spindle-shaped or oval-shaped cells, presumed to be suburothelial cells. Spherical cells with a diameter >35 μm were also avoided, except when specified below, and had the appearance of umbrella cells; smaller cells (<20 μm) were used for experiments.

Solutions. Nominally Ca^{2+}-free, HEPES-buffered solution contained (mM): NaCl, 132; KCl, 4.0; $MgCl_2$, 1.0; NaH_2PO_4, 0.4; HEPES, 10; glucose 6.1; Na pyruvate, 5.0, pH 7.4. Enzymes added for dissociation were: collagenase type-1 (1.0mg/ml Worthington Biochemical Corp, Lakewood, NJ, USA); hyaluronidase type I-S (0.25mg/ml) and type III (0.25mg/ml), trypsin inhibitor type-II-S (0.45mg/ml) and BSA (2.5mg/ml). During experiments, cells were superfused at 37°C with Tyrode's solution (mM): NaCl, 118; KCl, 4.0; $NaHCO_3$, 24; NaH_2PO_4,

0.4; $MgCl_2$, 1.0; $CaCl_2$, 1.8; glucose, 6.1; Na pyruvate, 5.0; gassed with 5% CO_2-95% O_2, pH 7.4. Low-osmolality solution was made by removing 88 mmol.l^{-1} NaCl from the superfusate for a final [Na]=59 mM; isosmolar, low-Na solution was made by equimolar replacement of the NaCl from Tyrode's solution with Tris-Cl. High osmolality solutions ([Na]=280 mM) were made by addition of 133 mM TrisCl to the normal Tyrode's solution or the isosmolar, low-Na solution. Na_2ATP, carbachol, Na_2UTP, thapsigargin and $GdCl_3$ were stored as aqueous stock solutions at least 1000-times more concentrated that used in experiments, except when Na_2ATP, carbachol and mannitol were used at concentrations ≥500 μM when they were added directly to Tyrode's solution. All chemicals were from Sigma Co except otherwise indicated.

Measurement of intracellular Ca^{2+}. Intracellular [Ca^{2+}], $[Ca^{2+}]_i$ was recorded using Fura-2, after incubation with the fluorochrome (5 μM) for 20-30 minutes at room temperature. When measured simultaneously with electrophysiological data, 100 μM K5 Fura-2 was added to the pipette filling solution, with the [EGTA] reduced to 50 μM [8]. Cells were excited at 340/380 nm at 50 Hz and fluorescence intensity recorded between 410-480nm. The Fura-2 signal was calibrated using solutions of varying [Ca^{2+}] in the absence of cells, yielding a K_d of 224 nM and β-factor of 10.9. Cell diameter was measured by projecting the cell in which intracellular Ca^{2+} was being measured onto a television monitor with a superimposed calibration grid.

Electrophysiological measurements. Ionic currents were measured with patch pipettes (3-4 MΩ) made from borosiliate glass and filled with a Cs-filling solution (mM): CsCl 20; aspartic acid, 110; MgCl2, 5.45; Na2ATP, 5.0; Na4GTP, 0.1; EGTA, 0.05; HEPES, 5.0, pH to 7.1 with

CsOH. An Axopatch 1-D system (Axon Instruments) was used for experiments and data recorded via an A/D converter (Digidata 1200, Axon Instruments) at 4 kHz, and filtered with a low-pass filter of cut-off frequency 2 kHz. A holding potential of -60 mV was used.

Ussing chamber experiments. The transepithelial potential difference (TEP) and the current required to clamp the TEP to zero, the short circuit current, were recorded from mucosal sheets using an Ussing chamber, membrane diameter 4.0 mm, and voltage-clamp system (DVC-1000 Dual Voltage Clamp, World Precision Instruments). The serosal (detrusor muscle facing) and apical sides of the sheets were noted and TEP was recorded as the apical potential with reference to the serosal face. Each face of the membrane was superfused by Tyrode's solution in a 0.5ml reservoir, maintained at 37°C, with constant perfusion. Ag/AgCl electrodes in 0.5M KCl salt bridges were placed in each reservoir to record TEP and also pass current to clamp TEP to 0 mV. Interventions were made by altering the superfusate to the serosal or apical bathing reservoirs.

Data analysis. Data are expressed as median values [25%, 75% interquartiles] as some data groups were not normally distributed. Differences between groups were examined using paired or unpaired non-parametric tests; the null hypothesis was rejected at $p<0.05$.

Results

Resting intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. When superfused with Tyrode's solution the resting $[Ca^{2+}]_i$ of urothelial cells was 135 nM [53, 265; n=61 cells). About 20% of cells generated spontaneous rises of $[Ca^{2+}]_i$, that in some cells occurred at a constant frequency and in others were more random. Experimental interventions were introduced only when such activity had terminated, except when interventions were specifically targeted to such activity. Thapsigargin (500 nM) always increased the resting $[Ca^{2+}]_i$, with a median change of 110 nM [84, 178; n=11], but Gd^{3+} had no significant effect ($\Delta[Ca^{2+}]_i$, 4 nM [6, 19; n=5]).

Action of low-Na, low osmolality solutions, effects of $Gd^{3+}$and thapsigargin. The low osmolality, low-Na solution generated cell swelling (cell diameter 113±7% control) and reversible intracellular Ca^{2+} transients with a median $\Delta[Ca^{2+}]_i$ of 192 nM [115, 464; n=42]. With some examples $[Ca^{2+}]$ remained elevated throughout the intervention (Figure Ai), whilst with others the $[Ca^{2+}]$ returned to the resting level even in the low osmolality solution (Figure Aii).

Figure 1B shows that the rise of $[Ca^{2+}]_i$ generated by the low-Na, low-osmolality solution was decreased in a time-dependent process by 100 μM $GdCl_3$: the decline of the Ca-transient in $GdCl_3$ was accompanied by its prolongation. Similar results were seen in five experiments, with a median attenuation to 28.5 % [12.9, 60.1; n=5] of the control value. In addition the recovery of the Ca^{2+} transient after removal of the $GdCl_3$ solution was largely reversible. Ca-transients were also greatly attenuated in low-osmolality, Ca-free solutions indicating that the source of Ca^{2+} was extracellular. By contrast thapsigargin (500 nM) had

no significant effect on the magnitude of the low-Na, low-osmolality Ca^{2+} transient ($\Delta[Ca^{2+}]_i$, 123 % [93, 141; n=10] of control).

Control experiments were performed to clarify if the rise of intracellular $[Ca^{2+}]$ was due to a change of osmolality or ionic composition of the solution. In a low-Na solution of normal osmolality (substituted with Tris-Cl) the $\Delta[Ca^{2+}]_i$ was significantly smaller ($\Delta[Ca^{2+}]_i$, 39 nM [3, 85; n=10]) implying that Na^+/Ca^{2+} exchanger is contributing only a minor fraction of the rise of $[Ca^{2+}]_i$ in the low-osmolality solution. Of interest was that in a high osmolality, normal-Na solution there was also an increase of $[Ca^{2+}]_i$ was ($\Delta[Ca^{2+}]_i$, 69 nM [42, 240; n=6]), intermediate between the low-osmolality solution and the isosmolar, low-Na solution. However, this intervention was not analysed further due to the relative variability of the changes; the control experiments are summarised in Figure 1C.

Action of exogenous ATP; thapsigargin and carbachol. ATP also generated large intracellular Ca^{2+} transients (figure 2), the median $\Delta[Ca^{2+}]_i$ was 670 nM [317, 982; n=26], that were unaffected by exposure to Ca-free solution for up to 200 seconds. In 15 cells ATP-dependent and low-osmolality, Ca-transients were elicited consecutively, after an intermediate return to Tyrode's solution; the low-osmolality Ca-transient was significantly smaller; 71% [34, 93] of the ATP transient. The intervention order was random in different cells and did not influence the relative size of the two transients.

Application of thapsigargin increased the resting $[Ca^{2+}]_i$ from 199 nM [123, 274; n=11] to 339 nM [260, 429]. Thapsigargin also reduced the magnitude of the ATP-dependent Ca-transient to 17% [13, 29; n=10] of control. Upon removal of thapsigargin, the resting

$[Ca^{2+}]_i$ recovered to normal levels and the ATP-dependent Ca-transients recovered towards their normal values. Pretreatment with carbachol (20 μM) prior to exposure to ATP had no effect on the Ca-transient. In six experiments the magnitude of the Ca-transient in the presence of carbachol was 100.2% [88.7, 104.2%] of that in ATP-containing solution alone.

Spontaneous activity and the action of carbachol. Carabachol exerted little effect on resting $[Ca^{2+}]_i$ in these cells; 20 μM carbachol increased $[Ca^{2+}]_i$ by 20 nM [10, 43; n=6]. Spontaneous Ca-transients were also recorded in 21 of a total of 65 cells studied. The magnitude of the transients (median $\Delta[Ca^{2+}]_i$, 798 nM [403, 1040, n=7] was similar to the ATP-evoked transients and for any given cell their frequency was very regular (Figure 3). The action of carbachol on these Ca-transients was also investigated. Carbachol (20 μM) had no significant effect on the magnitude of the transients (median $\Delta[Ca^{2+}]_i$, 868 nM [447, 929, n=7]). However, the agonist caused a small but significant reduction of their frequency: control 0.52/min [0.40,0.69] *vs* carbachol 0.44/min [0.31, 0.52]. In about half of the cells tested Ca-transients eventually disappeared in the presence of carbachol (Figure 3), although this occurred after a significant delay, the reason for which is not known.

Electrophysiological changes in urothelium cells. The pathway by which ATP altered the $[Ca^{2+}]_i$ was explored by measuring simultaneously membrane current and $[Ca^{2+}]_i$ during spontaneous or evoked Ca-transients. Figure 4A shows that a large inward current accompanied the rise of $[Ca^{2+}]_i$ following application of ATP. A phase-plot of the two signals (Figure 4B), whereby current is plotted as a function of $[Ca^{2+}]_i$, showed that current followed the $[Ca^{2+}]_i$ change. Figure 4C,D shows the same temporal relationship for a spontaneous Ca-transient. We interpret this, as ATP initially causing a rise of the $[Ca^{2+}]_i$

followed by membrane current generated secondarily due to this rise of $[Ca^{2+}]_i$ [9]. This relationship is typical of P2Y, rather than P2X receptor activation by ATP and this pathway was further given credence in three observations that UDP and UTP, both P2Y receptor agonists also generated a Ca-transient and inward current of comparable magnitude to ATP itself (Figure 4E).

Effect of ATP and carbachol on the electrophysiology of urothelium sheets. The transmembrane potential (TEP) under control conditions was -14.5 mV [-8.0, -30.0; n=26; apical *vs* serosal membrane] and the short-circuit current required to offset the TEP to 0 mV was 1.39 μA [0.89, 2.22] or 3.65 $\mu A.cm^{-2}$ [2.33, 5.81]. The Na-sensitivity was tested to demonstrate the preparation viability and Table 1 shows that decreasing the [Na] in the serosal bath to 29 mM increased both the TEP and SCC, whilst raising it to 280 mM had the opposite effect. ATP or carbachol added to the serosal chamber in the range 1-100 μM had little effect on TEP or SCC; however, higher ATP concentrations (0.5-1.0 mM) increased TEP and SCC. Conversely, carbachol at concentrations ranging from 1 μM to as high as 3 mM had no significant effect on TEP or SCC. Because of the high concentrations of agonists that were sometimes added 3mM mannitol was added to the serosal side as an osmotic control but also had no significant effect on TEP or SCC.

Discussion

Low-osmolality solutions and exogenous purines generate an increase of the intracellular $[Ca^{2+}]$, $[Ca^{2+}]_i$, in freshly-isolated urothelial cells. The urothelium/suburothelium contains several cells types, including specialised umbrella cells and suburothelial interstitial cells. An aim of this work is to characterise the different cell types and their potential contribution to transmitter release. This may be achieved by measuring the overall properties of the tissue, as with the Ussing chamber experiments, as well as the properties of component cells. In this study the non-umbrella component of urothelial cells was chosen. We excluded umbrella cells on the basis of cell size and spindle-shaped suburothelial cells but did not attempt any further subdivision.

Low-osmolality solutions have been used by many groups to cause cell swelling [12] and was also the case in these urothelial cells. However, the cellular pathways that mediate the rise of $[Ca^{2+}]_i$ were different: Gd^{3+} attenuated the responses due to low-osmolality solutions, whereas thapsigargin reduced the ATP-induced responses. Gd^{3+} are believed to block cation influx through stretch-activated membrane channels in many cell types, including epithelium and smooth muscle [13,14], and the ability of low-osmolality solutions to generate a large rise of $[Ca^{2+}]_i$ indicates the importance of the mechanism in these cells. Some experiments with a high-osmolality solution also generated significant Ca^{2+}-transients, supporting the hypothesis that changes to cell membrane stress underlay Ca^{2+} influx to generate the transient.

Several lines of evidence suggest that ATP generates Ca-transients through a P2Y, rather than a P2X-dependent mechanism: the lack of influence of Gd^{3+} or Ca-free solutions on

these Ca-transients; their attenuation by thapsigargin, an agent that blocks Ca^{2+} uptake into intracellular Ca-stores [15] and thereby limits subsequent release; a similar effect of purines such as UTP; and the fact that the rise of $[Ca^{2+}]_i$ occurred before the generation of membrane current. The significant rise of $[Ca^{2+}]_i$ by thapsigargin alone signifies the importance of intracellular Ca-stores in these cells. In contrast to P2X receptors, P2Y receptors elicit responses by modulating IP_3-dependent or cAMP-dependent intracellular pathways that are not initiated by transmembrane ionic currents, but may elicit subsequent opening of Ca^{2+}-dependent channels as occurs in suburothelial cells of the bladder wall [11]. This differentiation of hypoosmotic and ATP-induced pathways contrasts to other cells types where both interventions act through an IP_3-dependent mechanism [16-18].

Stretch of urothelial sheets has been shown to increase not only serosal ATP release but also alter transepithelial potential (TEP) and short-circuit current (SCC) and it may be that these two phenomena are linked. These experiments showed that ATP had a similar action on TEP and SCC, and others have also demonstrated an ATP-dependent increase of ATP release [3]. It may thus be postulated that a rise of intracellular $[Ca^{2+}]_i$ in urothelial cells mediates ATP-release which could underlie the autocrine effect of this transmitter.

In these experiments the muscarinic agonist carbachol generated significant, but only very small increases of $[Ca^{2+}]_i$ in these cells. This is in contrast to reports from cultured rat and human urothelial cells where more substantial Ca-transients were recorded [8,19]. However, it should be noted that in one of the reports [8] no muscarinic agonist responses were recorded in about 50% of cells. It is not possible to reconcile these opposing findings,

except that cell culture may have altered the profile of functional receptors or that a subset of cells was preferentially chosen for these experiments that was not reflected in the cell profile that were viable in culture. Furthermore, in our experiments carbachol exerted no effect on the electrophysiological properties (TEP and SCC) of urothelial sheets, suggesting it did not regulate ion transport in this preparation.

A significant proportion of urothelial cells also generated regular spontaneous Ca^{2+} transients as illustrated in Figures 3 and 4 and indicates Ca^{2+} release from intracellular stores. The transients were large and regular in frequency – about 0.5 per minute. The lag between onset of the Ca transient and the development of inward current, suggests that the current is activated by an increase of the $[Ca^{2+}]$. Although the ionic nature of the current was not measured in these experiments, similar observations have been made in suburothelial interstitial cells, where a Ca^{2+}-activated Cl^- current is the dominant membrane conductance [11]. Carbachol had a small decelerating influence on the transients and in several cells disappeared in the presence of the agonist, however it remains to be determined if this was a coincidence or a true effect and such Ca-transients rarely reappeared after carbachol removal. However, the presence of such Ca-transients will increase the average $[Ca^{2+}]$ and thus upregulate any Ca^{2+}-dependent cellular activity.

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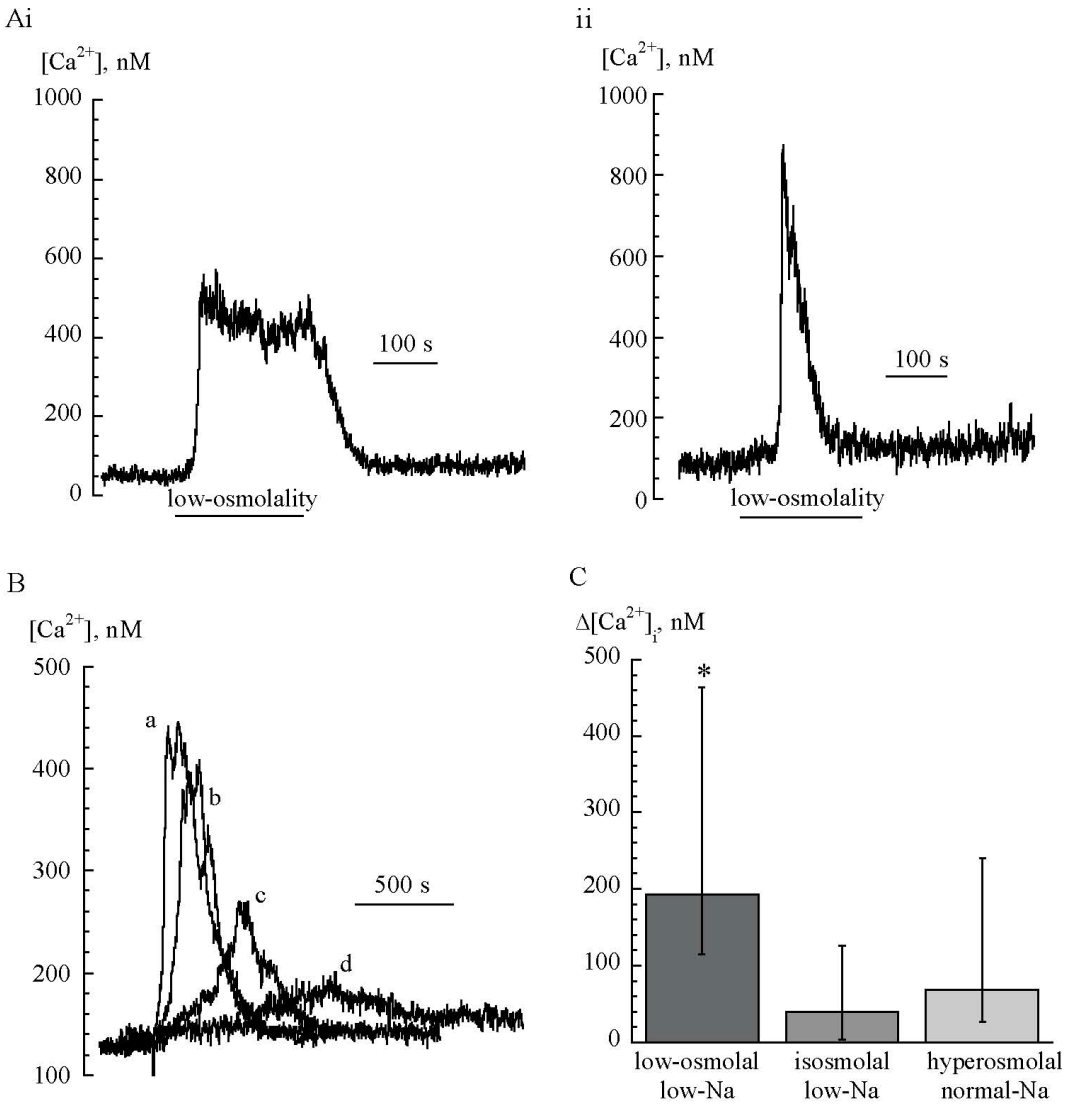
Table 1. The effect of ATP, carbachol (0.5–1.0 mM) and control solutions on changes to urothelial transepithelial potential (ΔTEP, mV) and short-circuit current (ΔSCC, μA). A negative ΔTEP denotes a larger value, whilst a positive SCC equates to a greater current required to clamp the membrane to 0 mV. All interventions on the serosal face. Median values [25%, 75% interquartiles]. *p<0.05 compared to control. *n*=number of experiments.

Solution	ATP	carbachol	29 mM Na	280 mM Na	mannitol
	n=23	*n*=10	*n*=14	*n*=6	*n*=6
ΔTEP, mV	-2.3	0.04	-3.9	12.2	0.04
	[1.8, 3.8]	[-1.0, 1.0]	[-2.0, -5.5]	[8.6, 19.5]	[-0.1, 0.1]
ΔSCC, μA	0.30	-0.02	0.28	-3.21	0.01
	[0.12, 0.50]	[-0.13, 0.11]	[0.21, 0.52]	[-1.72, -3.71]	[-0.04, 0.03]

Figure legends

Figure 1.



Intracellular Ca-transients generated by exposure to a low-osmolality solution. Part A i and ii: Examples of Ca-transients. Part B: Effect of 100 μM $GdCl_3$ on low-osmolality Ca-transients: a, control; b, 550 s; c, 1500 s; d, 3150 s after addition of $GdCl_3$. Part C: The increase of intracellular $[Ca^{2+}]$ in response to solutions of low-osmolality, low-Na; normal osmolality, low-Na; hyperosmolality, normal-Na; *$p<0.05$ low osmolality low-Na *vs* isosmolarity, low-Na

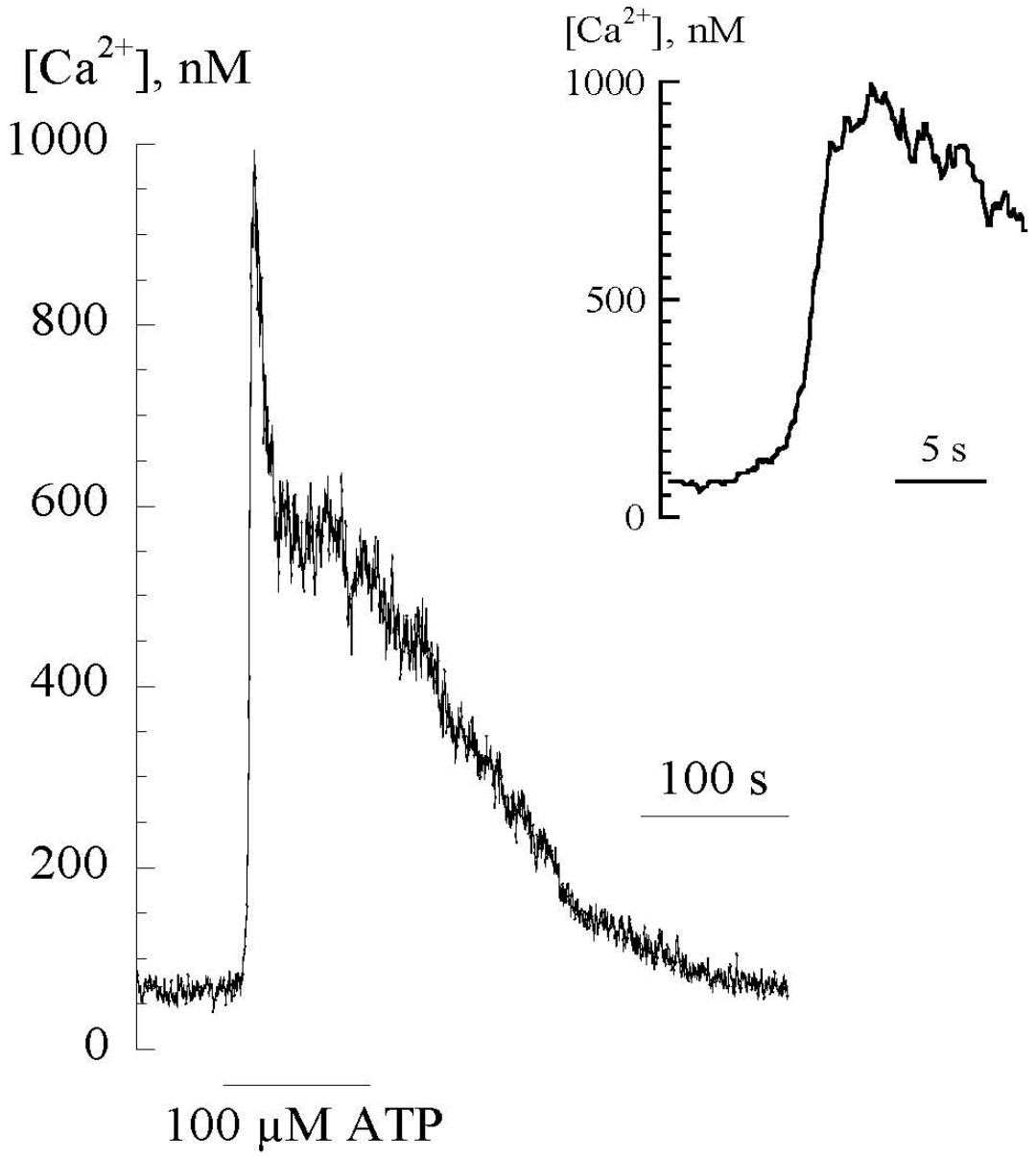


Figure 2. Ca-transient generated by exposure to 100 µM ATP. The inset shows the initial phase on a faster time-base.

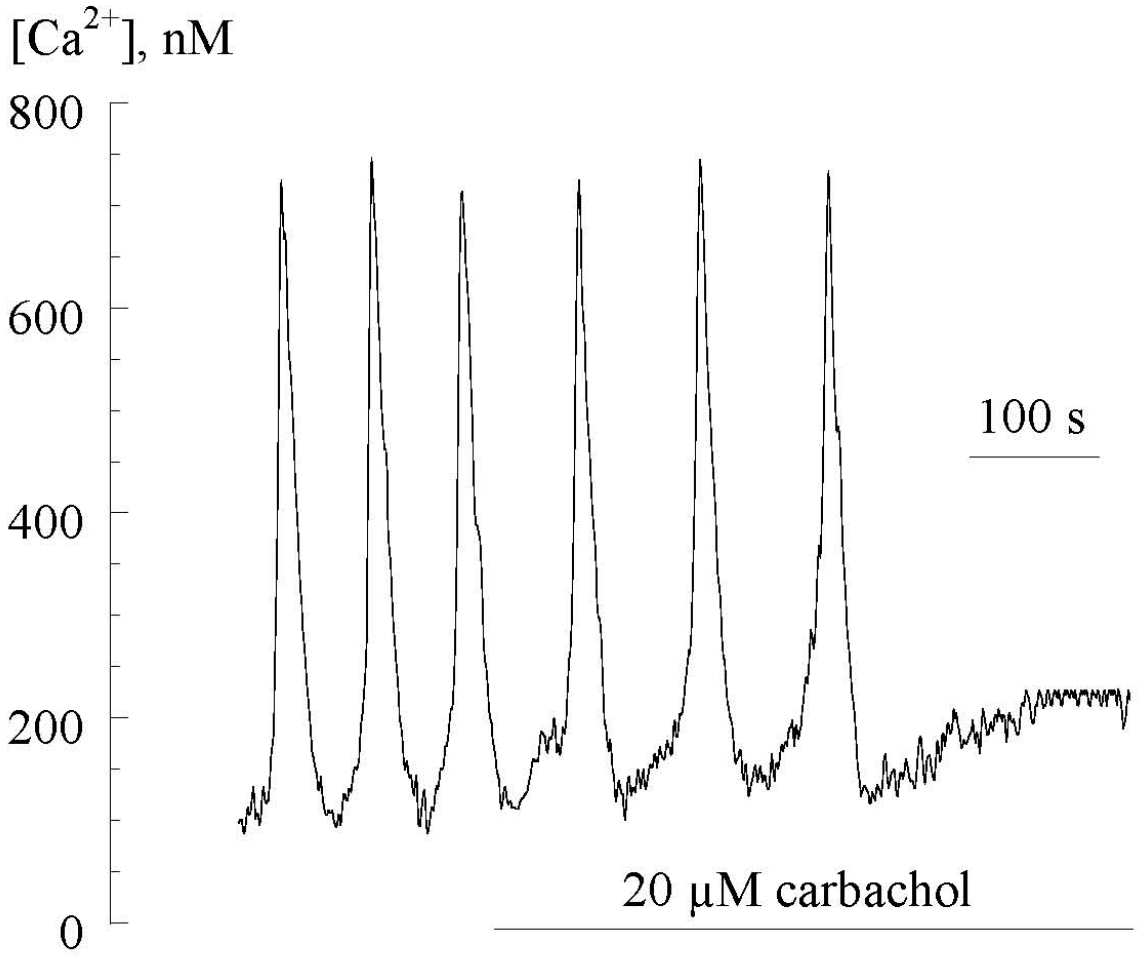
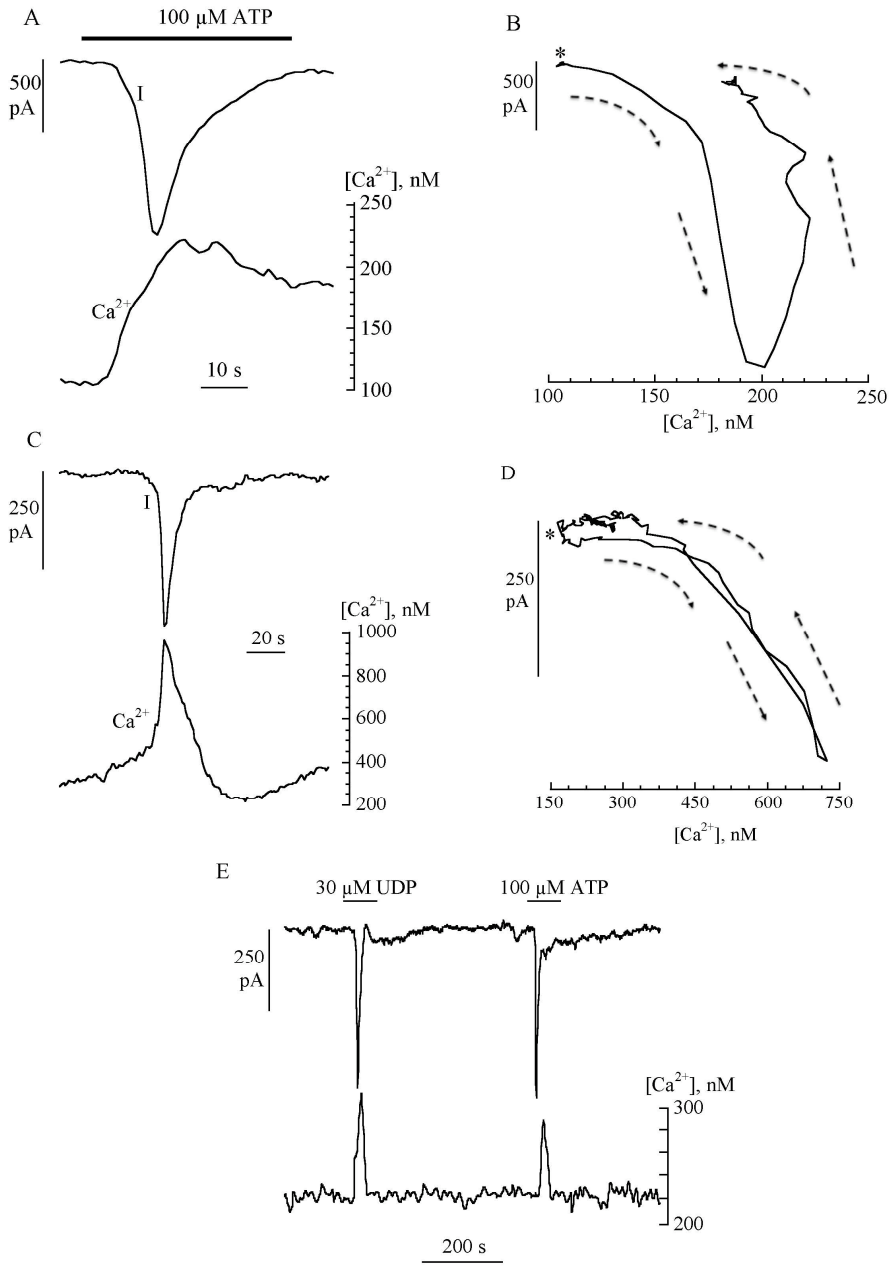


Figure 3. Spontaneous Ca-transients. Carbachol (20 μM) was added during the period indicated.

Figure 4.



Simultaneous measurement of membrane current and intracellular [Ca^{2+}]. A: Membrane current and intracellular [Ca^{2+}] after exposure to 100 µM ATP. B: Phase-plot of the dependence of current on [Ca^{2+}]. The arrows denote time from the beginning of the recordings marked by the large star. C: Membrane current and intracellular [Ca^{2+}] during a spontaneous event. D: Phase-plot of the data in part C. E. The action of UDP and ATP on membrane current and intracellular [Ca^{2+}].