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Cyclic AMP increases cytoplasmic free calcium in renin-secreting cells from rat kidney

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Abstract. The renin-secreting juxtaglomerular cells (JGC) in the media of the afferent arteriole at the vessel pole are the major source of circulating renin. The control of renin secretion is complex with increases in cAMP being the major stimulus and increases in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) being inhibitory. We measured $[Ca^{2+}]_i$ in the afferent arteriole from mostly JGC. Manoeuvres that increase cAMP (e.g. isoproterenol) or dibutyryl-cAMP elicited an increase in $[Ca^{2+}]_i$ which was ~40% of that induced by angiotensin II (3 nmol/l). The Ca^{2+} response occurred in 50–90% of the cases, and increasing the stimulus increased responder frequency but not response size. The response was (almost) abolished by removal of extracellular Ca^{2+}, prevented by inhibitors of store-operated Ca^{2+} channels (Gd^{3+} and 2-aminoethoxydiphenyl-borate), but was unaffected by isradipine or protein kinase A inhibitors. It was not produced by an activator of EPACs (exchange protein activated by cAMP) and was not accompanied by changes in membrane potential. The data suggest that in rat JGC, cAMP, perhaps directly, activates store-operated Ca^{2+} channels to increase $[Ca^{2+}]_i$. One could speculate that this increase in $[Ca^{2+}]_i$ serves to finely adjust the stimulating effect cAMP-increasing signals on the renin-angiotensin system.

Key words: Afferent arteriole — Ca^{2+} concentration — Cyclic AMP — Kidney — Renin-secreting cells

Abbreviations: 2-APB, 2-aminoethoxy diphenylboran; ANG II, angiotensin II; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CPA, cyclopiazonic acid; IBMX, 3-isobutyl-1-methylxanthine; ISO, isoproterenol; JGC, juxtaglomerular cells; PDE, phosphodiesterase; SERCA, sarcoplasmic Ca^{2+}-ATPase; SOCC, store-operated Ca^{2+} channels.

Introduction

The activity of the renin-angiotensin-aldosterone system (RAAS) is governed by the rate of renin secretion from the juxtaglomerular (renin-secreting) cells (JGC) (Hackenthal et al. 1990; Persson et al. 2003). These cells are located in the media of the afferent arteriole near the entrance of the vessel into the glomerulus (Hackenthal et al. 1990). The regulation of renin synthesis and secretion is complex and several extra- and intrarenal mechanisms contribute to a flexible control according to the needs of the body (Kurtz 1989; Hackenthal et al. 1990; Osswald and Quast 1995; Schweda and Kurtz 2004).

At the cellular level, important renin-stimulating mechanisms like an increase in sympathetic tone (*via* noradrenaline and β_1 receptors) or in prostaglandin E_2, prostacyclin, dopamine etc. converge to activate adenylyl cyclase and increase cAMP (reviews: Hackenthal et al. 1990; Schweda and Kurtz 2004). Interruption of this signalling chain by deletion of the G-protein subunit Gsα (which stimulates adenylyl cyclase) demonstrated that cAMP is the major stimulus for renin synthesis and secretion (Chen et al. 2007). The mechanism by which cAMP increases renin secretion remains unknown. On the other hand, increases in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in JGC decrease the rate of renin secretion (Churchill 1985; Hackenthal and Taugner 1986). This "inverse" Ca^{2+} dependence is highly unusual for

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an exocytotic process and has been termed the "Ca^{2+} paradox of renin secretion". Several mechanisms have been advanced to explain this phenomenon (reviews: Schweda and Kurtz 2004; Schweda et al. 2007). Very recently, it has been shown that increases in $[Ca^{2+}]_i$ inhibit Ca^{2+}-sensitive adenylyl cyclase subtypes expressed in mouse JGC and blunt the increase in cAMP and in renin secretion induced by isoproterenol (ISO) or forskolin (Grünberger et al. 2006; Ortiz-Capisano et al. 2007a; 2007b). Hence, a major mechanism underlying the Ca^{2+} paradox of renin secretion is a Ca^{2+}-mediated inhibition of cAMP synthesis in JGC.

A certain level of complexity is reached, however, if one considers that increases in cAMP may, in turn, affect $[Ca^{2+}]_i$ since many proteins involved in cell Ca^{2+} homeostasis are phosphorylated by protein kinase A (PKA). In (vascular) smooth muscle, the relationship between an increase in cAMP is mostly accompanied by a decrease or no change in $[Ca^{2+}]_i$; only rarely, an increase was observed (review: Karaki et al. 1997; Takuwa et al. 1988). In mouse JGC, dialysis with cAMP (50 µmol/l) did not affect resting $[Ca^{2+}]_i$ but prevented angiotensin (ANG) II-induced $[Ca^{2+}]_i$ oscillations (Kurtz and Penner 1989). Similarly, in the rabbit afferent arteriole, increasing cAMP by various manoeuvres did not affect resting $[Ca^{2+}]_i$ but blunted the noradrenaline-induced Ca^{2+} response (Kornfeld et al. 2000).

In this study we report that in the rat afferent arteriole, increases in cAMP induce an increase in $[Ca^{2+}]_i$ by opening store-operated Ca^{2+} channels (SOCCs). The experiments were performed in vessels prepared from rats kept on a NaCl-depleted diet; under these conditions the number of JGC is greatly increased and the cells contributing to the signal near the vessel pole are mostly JGC (Hackenthal et al. 1990; Leichtle et al. 2004). Hence, one might speculate that in rat JGC, the stimulating action of cAMP on renin secretion is fine-tuned by a concomitant increase in $[Ca^{2+}]_i$ which reduces secretion, resulting in a more balanced stimulation of the RAAS.

Materials and Methods

Animals

Animal experimentation was conducted in accordance with the German Law on the Protection of Animals. To induce NaCl-depletion, Sprague-Dawley rats (250–500 g; Charles River, Germany) were treated once with furosemide (10 mg/kg, i.p.) and kept on a NaCl-depleted diet (0.015% Na^+, 0.015% Cl^-, C1036, Altromin, Germany) for at least 2 weeks after furosemide application. Control animals were not treated with furosemide and were kept on a standard (0.2% Na^+, C1324, Altromin) or Na^+-rich diet (3% Na^+, C1051, Altromin).

Preparation of glomeruli

Animals were killed by cervical dislocation, exsanguinated and the kidneys removed. A kidney was immediately transferred in a HEPES-buffered physiological salt solution (PSS) containing (in mmol/l): NaCl 142, KCl 2.8, $MgCl_2$ 1, $CaCl_2$ 1, D(+)-glucose 11, buffered with HEPES (10 mmol/l) and titrated to pH 7.4 with NaOH at 37°C. The kidney was decapsulated, cut longitudinally into two halves and the isolated cortex was minced with a razor blade. Glomeruli were prepared by a modification of the method published by Kurtz and Penner (1989) and Metzger and Quast (1996), as described by Russ et al. (1999). The minced tissue was incubated for 50 min in 20 ml PSS with 20 mg collagenase A (Roche Diagnostics, Germany) at 37°C under gentle shaking, then passed through stainless steel sieves of mesh 200 and 125 µm and the final material collected on a 63 µm sieve. Microscopic inspection showed that the preparations consisted mainly of glomeruli and some contamination with tubular fragments; in about 10% of the cases, a remnant of the afferent arteriole up to 100 µm in length remained attached to the glomerulus (Russ et al. 1999, Fig. 1; Laske-Ernst et al. 2008).

Fluorescence measurements

For measurement of $[Ca^{2+}]_i$, the dye fura-2 was used. Epifluorescence was measured with an inverted microscope (DIAPHOT 300, Nikon, Japan), equipped with a ×100 fluor oil-immersion objective (numerical aperture, 1.3; Nikon) and a Image Master fluorescence imaging system (Photon Technology International, Inc., NJ, U.S.A). Excitation wavelengths were set to 340 and 380 nm and the microscope was equipped with a dichroic mirror (430DCLPO2, Omega Optical, Inc., VT, USA) and an interference barrier filter (510WB40, Omega Optical). Cells were loaded with the indicator by incubation of glomeruli in PSS with 16 µmol/l fura-2 AM (acetoxymethyl; Invitrogen or Biotrend, Germany), the membrane-permeable AM ester of fura-2, and 0.05% (w/v) pluronic F127 for 60 min at room temperature. After washing with PSS for 30 min fluorescence measurements were performed by selecting an area of about 10–20 cells from the afferent arteriole near the entrance into the glomerulus (Laske-Ernst et al. 2008, Fig. 1). These cells were clearly distinguishable from smooth muscle cells which have a spindle-like appearance and a spiral arrangement around the vessel (Bührle et al. 1984; Hackenthal et al. 1990); instead, their roundish shape suggested them to be renin-secreting cells (Russ et al. 1999; Leichtle et al. 2004; Laske-Ernst et al. 2008). About 38 pictures resulting in 19 ratios *per* minute were recorded and stored. During washout periods sampling frequency was reduced to avoid dye bleaching. In intact afferent arterioles, calibration of the fura-2 signal is difficult

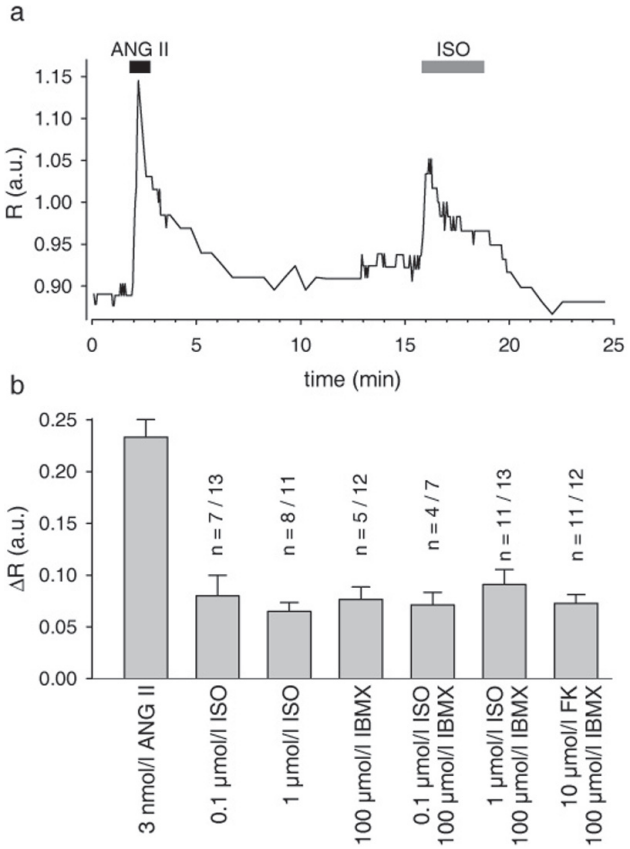

Figure 1. ISO and IBMX increase fura-2 fluorescence ratio R in the proximal part of the afferent arteriole from rat kidney. **a)** Original trace showing the response to ANG II (3 nmol/l; 1 min) followed by a washout period and superfusion of ISO (1 µmol/l; 3 min). **b)** Peak increases in R to stimulation by ANG II as compared to ISO, IBMX, and forskolin (FK) (partly in combination). All vessels responded to ANG II whereas the response to cAMP-increasing agents was sometimes lacking. The bars show the mean response of the responding vessels only and above the bars, the numbers of the responding preparations/all preparations are given ($n = 44$ for ANG II). The responding preparations showed a similar peak increase in R which was ~35% of that to ANG II. Increasing the strength of the stimulus by increasing the concentration or adding IBMX increased the rate of responding vessels but left response size unchanged.

(Fowler et al. 1996; Kornfeld et al. 1997; Russ et al. 1999; Laske-Ernst et al. 2008), therefore the fluorescence ratio R of the fura-2 fluorescence at the two wavelengths (340 nm/380 nm) is given (arbitrary units, a.u.). The calcium concentration in the preparation is given in Laske-Ernst et al. (2008).

During the experiment arterioles were permanently superfused; solution influx into the experimental chamber was driven by gravity and excess solution removed continuously. Flow artefacts are highly unlikely since switching between two reservoirs with the same solution was without effect. In solutions with high or low Ca^{2+}, osmolarity was maintained by reducing NaCl concentration; in the low Ca^{2+}-solution, $MgCl_2$ was increased from 1 to 2 mmol/l.

In general, functional integrity of the cells was assessed by application of 3 nmol/l ANG II for 1 min and the response was used as the internal standard for a second (test) stimulation. Response parameters were the response frequency and the maximal increase fluorescence ratio R or, in some cases, the area under the fluorescence ratio R *versus* time curve.

To monitor changes in membrane potential, the fluorescent oxonol dye $DiBAC_4(3)$ (bis-(1,3-dibutylbarbituric acid) trimethine oxonol; Invitrogen; Bräuner et al. 1984) was used. Excitation wavelength was set to 488 nm and the microscope was equipped with a dichroic mirror (DM 505, Nikon) and a long wave pass barrier filter (BA 520, Nikon; for details see Russ et al. 1999).

Substances

Most substances used were from Sigma (Germany). Isradipin was from Novartis (Suisse), 2-aminoethoxy diphenylboran (2-APB) was from Tocris (Germany), thapsigargin was from Alomone (Germany), dibutyryl-cAMP (db-cAMP) and 8-pCPT-2'-O-Me-cAMP were from Biolog (Germany), KT 5720 was from Calbiochem (Germany) and forskolin was from Sanofi-Aventis (Germany). Drugs normally were prepared as 10 mmol/l stock solution in ethanol/DMSO (1 : 1).

Calculations and statistics

Fluorescence ratio R data were successfully tested for normal distribution using the Kolmogorov-Smirnov test. This allows presentation of the results as means ± SEM and statistical comparison using the *t*-test. Response frequency is compared with the Fisher Exact test. A difference was assumed significant if $p \leq 0.05$.

Results

Manoeuvres that increase cAMP also increase $[Ca^{2+}]_i$

JGC respond to ANG II with depolarisation and an increase in $[Ca^{2+}]_i$ (Kurtz and Penner 1989). After two subsequent stimulations with ANG II (3 nmol/l; 1 min), the second Ca^{2+} response was between 80–100% of the first if a recovery period of >10 min was allowed between the two stimuli (not shown). Fig. 1a illustrates the responses of the fura-2 fluorescence ratio R from a vessel stimulated first with ANG II (3 nmol/l; 1 min) and then with ISO (1 µmol/l, 3 min).

Surprisingly, ISO also increased R; however, the response to ISO was smaller than that to ANG II. In 11 such experiments, 8 vessels (~75%) responded to ISO (1 μmol/l) and the peak increase in R was 37 ± 8% (responders only; $n = 8$) of that to ANG II (Fig. 1b). Stimulation by a lower concentration of ISO (0.1 μmol/l) decreased the number of responders somewhat ($p > 0.05$) to 50% but left the magnitude of the response unchanged (46 ± 26% of that to ANG II; responders only; $n = 3$). Similar observations were made when ISO was used without a prior stimulation with ANG II (pooled data shown in Fig. 1b).

To examine whether the ISO-induced increase in Ca^{2+} response was mediated *via* β-adrenoceptors, arterioles were stimulated in the absence (first stimulus) and presence (second stimulus) of the β-blocker, propranolol (1 μmol/l), or the α-blocker, prazosine (0.1 μmol/l). Propranolol totally abolished the response ($n = 4$) whereas prazosine left the response unaltered ($n = 6$), showing that the response was indeed mediated *via* the activation of β-adrenoceptors. Experiments were also performed with ISO in the presence of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 100 μmol/l). IBMX alone elicited a Ca^{2+} response in ~40% of the cases and, when applied together with ISO, increased the frequency but not the size of the response (Fig. 1b). Even though at 0.1 μmol/l ISO this increase is small (~60% responder) at ISO (1 μmol/l) + IBMX (100 μmol/l), the responder rate was ~85% (Fig. 1b; $p < 0.05$ if compared with IBMX alone).

The direct stimulator of adenylyl cyclase, forskolin (10 μmol/l) also elicited a Ca^{2+} response. If applied in combination with IBMX, 90% of the vessels responded ($p < 0.05$ if compared with IBMX alone) and the peak increase in R was of the usual size (Fig. 1b). In addition, the membrane-permeant cAMP analogue, db-cAMP (1 mmol/l) increased $[Ca^{2+}]_i$. Fig. 2a shows a trace from an arteriole stimulated first with ISO + IBMX and then with db-cAMP + IBMX. All vessels responding to the first stimulus also responded to db-cAMP in the presence or absence of IBMX (Fig. 2c). Stimulation by forskolin or db-cAMP was only partially reversible (Fig. 2a).

In vascular smooth muscle, most effects of cAMP are mediated *via* activation of PKA. Fig. 2b shows that the ISO + IBMX-induced increase in $[Ca^{2+}]_i$ was not affected by the PKA inhibitors N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89; 1 μmol/l) (Chijiwa et al. 1990). Identical results were obtained with H89 (10 μmol/l) and with KT 5720 (1 μmol/l; Ellershaw et al. 2002) (Fig. 2c). This indicated that PKA was not involved in the signalling chain linking the increase in cAMP to the increase in Ca^{2+}. cAMP can also act by activating "exchange proteins directly activated by cAMP" (EPACs; Kang et al. 2003). The specific activator of EPAC, 8-pCPT-2'-O-Me-cAMP (Kang et al. 2003), at the concentration of 200 μmol/l, did not increase R (Fig. 2c), demonstrating that also EPACs were not involved in the Ca^{2+}-increasing effect of cAMP.

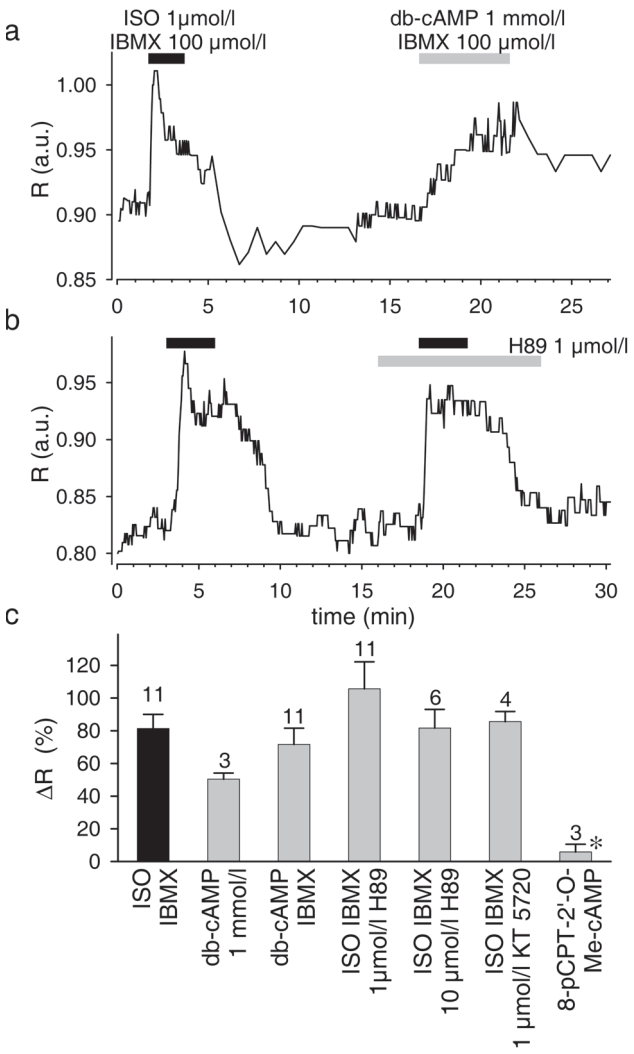

Figure 2. Pharmacological properties of the cAMP-induced fura-2 fluorescence ratio R increase. A double stimulation protocol with ISO + IBMX as the first (control) stimulus was used and only experiments with arterioles responding to ISO + IBMX were continued. **a)** Trace showing that the membrane-permeant cAMP analogue db-cAMP (1 mmol/l) (+ IBMX) also elicited the Ca^{2+} transient. **b)** Trace showing that the PKA inhibitor H89 (1 μmol/l) did not affect the response to ISO + IBMX. **c)** Summary of these experiments. The peak Ca^{2+} increase (ΔR) of the second (test) stimulus is expressed in % of that of the first (ISO + IBMX). See text for details. There were no significant differences between the responses with the exception of that to the EPAC stimulator, 8-pCPT-2'-O-Me-cAMP. The number of experiments is shown above the bars.

Source of Ca^{2+} for the cAMP-induced transient

First it was examined whether the Ca^{2+} transient originated from Ca^{2+} influx. Therefore, the responses to ISO (1 µmol/l) + IBMX (100 µmol/l) were compared in Ca^{2+}-containing and Ca^{2+}-free solutions using the double stimulation protocol.

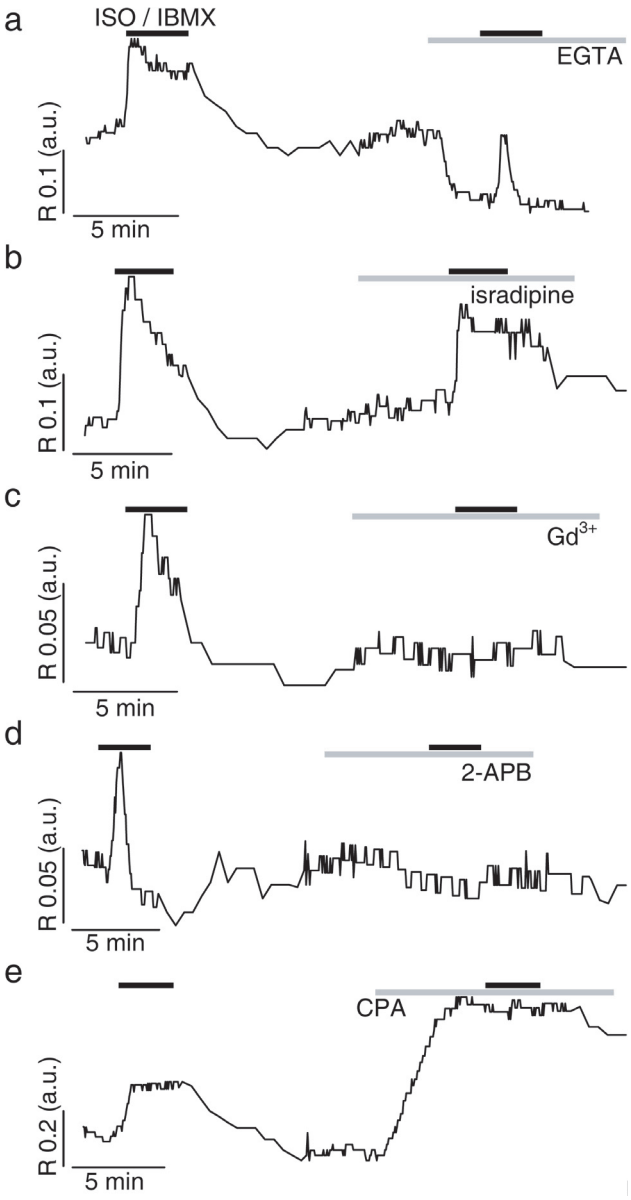

Figure 3. Characterisation of the cAMP-induced Ca^{2+} influx. A double stimulation protocol was used with ISO + IBMX as the first and the second stimulus. Traces show the effects of Ca^{2+} withdrawal (0 Ca^{2+} added, EGTA (5 mmol/l)) (a), isradipine (100 nmol/l) (b), Gd^{3+} (50 µmol/l) (c), 2-APB (30 µmol/l) (d), and the SERCA inhibitor, cyclopiazonic acid (CPA, 10 µmol/l) (e). The mean values of the respective experimental series are given in Tab. 1.

Control experiments showed that with two subsequent stimulations in the presence of Ca^{2+}, the second response was 81 ± 7% of the first (n = 4) when the area under the curve (AUC) was compared. However, when the second simulation was performed in Ca^{2+}-free solution (0 Ca^{2+} added, 5 mmol/l EGTA), the response was dramatically reduced (Fig. 3a) to 10 ± 2% of the second stimulus in the presence of Ca^{2+} (n = 9). This is in contrast to double stimulation with ANG II where Ca^{2+}-free conditions reduce the response of the second stimulus to 44 ± 6% of that in the presence of Ca^{2+} (n = 7). Collectively the data show that Ca^{2+} influx from outside carries >80% of the Ca^{2+} response to the cAMP increase and that Ca^{2+} release from stores contributes – if at all, only very little.

In order to characterise the cAMP-induced Ca^{2+} influx it was first examined whether dihydropyridine-sensitive (L-type) Ca^{2+} channels contributed to the Ca^{2+} transient. Fig. 3b illustrates that the response to ISO + IBMX was not affected by isradipine (100 nmol/l). This was confirmed in 11 experiments (Tab. 1) and ruled out a major contribution of L-type Ca^{2+} channels to the cAMP-induced Ca^{2+} influx. In addition, experiments using the membrane potential-sensitive dye $DiBAC_4(3)$ (Bräuner et al. 1984) gave no change in fluorescence in response to 1 µmol/l ISO + 100 µmol/l IBMX (n = 10) whereas hyperpolarising agents like the potassium channel opener levcromakalim decreased (Russ et al. 1999) and depolarising agents (ouabain, increases in the extracellular potassium concentration or ANG II) increased fluorescence. Therefore, these experiments show that stimulation by ISO + IBMX does not affect membrane potential, an observa-

Table 1. Inhibition of a second stimulation with ISO (1 µmol/l) and IBMX (100 µmol/l). As the peak value shown in Fig. 1b and 2c does not reflect the shortening of the response visible e.g. in Fig. 3a, we used the area under the curve (AUC_2) in % of the control stimulus (AUC_1) for quantification of the Ca^{2+} influx. Experiments were performed as shown in Fig. 3.

Substance	c_s (µmol/l)	AUC_2 (% AUC_1)	n
control	–	81	4
Ca^{2+} free (5 mmol/l EGTA)	–	8 ± 2***	9
isradipine	0.1	81 ± 7	11
Gd^{3+}	50	0	7
2-APB	30	0	5
CPA	10	26 ± 19*a	4
thapsigargin	1/0.1	< 0[b]	10
$[Ca^{2+}]_o$	20×10^3	88 ± 10	4

c_s, concentration of substance; n, number of experiments; [a] from 4 experiments 2 gave response 0, 1 with 22 % and the other with 82 %; [b] a slight decrease in $[Ca^{2+}]_i$ by 4 ± 1% was observed; * p = 0.05, *** $p < 0.001$.

tion corroborated by whole-cell current- and voltage-clamp experiments (Stehle, personal communication). Hence, a contribution of other depolarisation-activated Ca^{2+}-permeable channels to the response to cAMP is unlikely. In order to examine whether SOCCs were involved, the effects of the SOCC inhibitors, Gd^{3+} and 2-APB (Clapham et al. 2005; Parekh and Putney 2005) were examined. Fig. 3c and d show that Gd^{3+} (50 µmol/l) or 2-APB (30 µmol/l), applied prior to the second stimulus, abolished the response (Tab. 1). In contrast, the SOCC blockers had only a limited effect on the ANG II-induced Ca^{2+} increase (reduction to 67 ± 2% (Gd^{3+}; $n = 6$) and 87 ± 18% (2-APB; $n = 4$)).

The experiments in Ca^{2+}-free solution (Fig. 3a) suggested that the Ca^{2+} release from stores contributed little to the cAMP-induced Ca^{2+} response. To further support this conclusion the inhibitors of the sarcoplasmic Ca^{2+}-ATPase (SERCA), cyclopiazonic acid (CPA) and thapsigargin, were applied; the idea was that these inhibitors should deplete the stores and leave the cAMP response unaffected. Fig. 3e shows that CPA (10 µmol/l) led to a large increase in $[Ca^{2+}]_i$ reflecting the inability of the sarcoplasmic reticulum to accumulate Ca^{2+}; however, further stimulation with ISO + IBMX left $[Ca^{2+}]_i$ unaltered. In four experiments, the responses were heterogeneous giving a strongly reduced mean response (Tab. 1). Thapsigargin (1 µmol/l) induced an even larger increase in $[Ca^{2+}]_i$; stimulation with ISO + IBMX now induced a small decrease in $[Ca^{2+}]_i$ (Tab. 1). In order to examine whether the high $[Ca^{2+}]_i$ induced by the SERCA inhibitors inhibited the response to ISO + IBMX, the double stimulation was used. The first stimulus was applied in the presence of 1 mmol/l Ca^{2+}; then extracellular Ca^{2+} was increased to 20 mmol/l which increased $[Ca^{2+}]_i$ to a level slightly inferior that produced by the SERCA inhibitors. A second stimulation under these conditions gave a response identical to the control (Tab. 1).

Regional specificity of the Ca^{2+} response

The experiments presented here were performed near the entrance of the afferent arteriole into the glomerulus, i.e. the region where the renin-secreting cells are concentrated. In addition, the vessels were prepared from NaCl-depleted rats in order to maximise the number of renin-secreting cells (Taugner and Hackenthal 1989). We have indeed found that, the vast majority of cells that we observe under these conditions at the vessel pole are renin-positive (Leichtle et al. 2004). In the more proximal parts of the afferent arteriole, however, smooth muscle cells predominate and the responses to stimulation by ISO + IBMX were compared in the two regions (distal: 0–20 µm, proximal: 60–100 µm from the entrance into the glomerulus). In 17 experiments neither the peak of the $[Ca^{2+}]_i$ transient nor the rate of responding cells varied significantly with the distance to the vessel pole. Similar experiments were performed with vessels from rats fed with a NaCl-rich (4% NaCl) diet for 2 weeks. 23 preparations were compared in the distal and the proximal region and peak increases and responder rate were not different from the results obtained with vessels from NaCl-depleted rats. However, the response to ISO + IBMX was not observed in larger vessels, e.g. interlobular arteries ($n = 2$). Fig. 4 shows the results obtained from interlobular artery from which an afferent arteriole branches off. Whereas a response was elicited in the afferent arteriole no response was seen in the larger artery proximal and distal from the branching point.

Discussion

Isolated afferent arterioles

The experiments reported here were performed in isolated afferent arterioles. An advantage of this preparation is that the tissue remains essentially intact. This is of importance, as the cells in this preparation are strongly coupled by gap junctions (Kurtz and Penner 1989; Russ et al. 1999; Leichtle et al. 2004). A disadvantage of the preparation is the absence of an intravascular pressure which results in a negative membrane potential (from –60 to –65 mV (Kurtz and Penner 1989; Leichtle et al. 2004)) and low values of $[Ca^{2+}]_i$ (Laske-Ernst et al. 2008). Furthermore, the afferent arteriole is composed of smooth muscle cells and JGC and, at least in principle, one cannot be certain from which cell type the signal arises. However, these two cell types mutually transform into each other according to the demands of the body (Cantin et al. 1977; Hackenthal et al. 1990; Sequeira Lopez et al. 2001; Sequeira Lopez et al. 2004). Therefore, we have increased the number of JGC by NaCl-depleted diet and limited the region of interest to the most distal part of the arteriole where JGC predominate (Hackenthal et al. 1990; Leichtle et al. 2004) such that the signal essentially reflects $[Ca^{2+}]_i$ in JGC. On the other hand, the data in Fig. 4 showed that different parts of the afferent arteriole responded in the same way to ISO + IBMX whereas larger arteries further upstream did not show this response. This difference may reflect the common origin of JGC and afferent arteriole smooth muscle cells from the metanephric mesenchyme (Sequeira Lopez et al. 2001) in contrast to the smooth muscle cells in other vessels. In addition, the coupling by gap junctions limits independent responses of the two cell types.

cAMP induces a Ca^{2+} transient in afferent arterioles

This study has shown that stimulating the synthesis of cAMP (by ISO, forskolin), inhibiting its degradation (by IBMX) and application of db-cAMP, a membrane-permeant cAMP

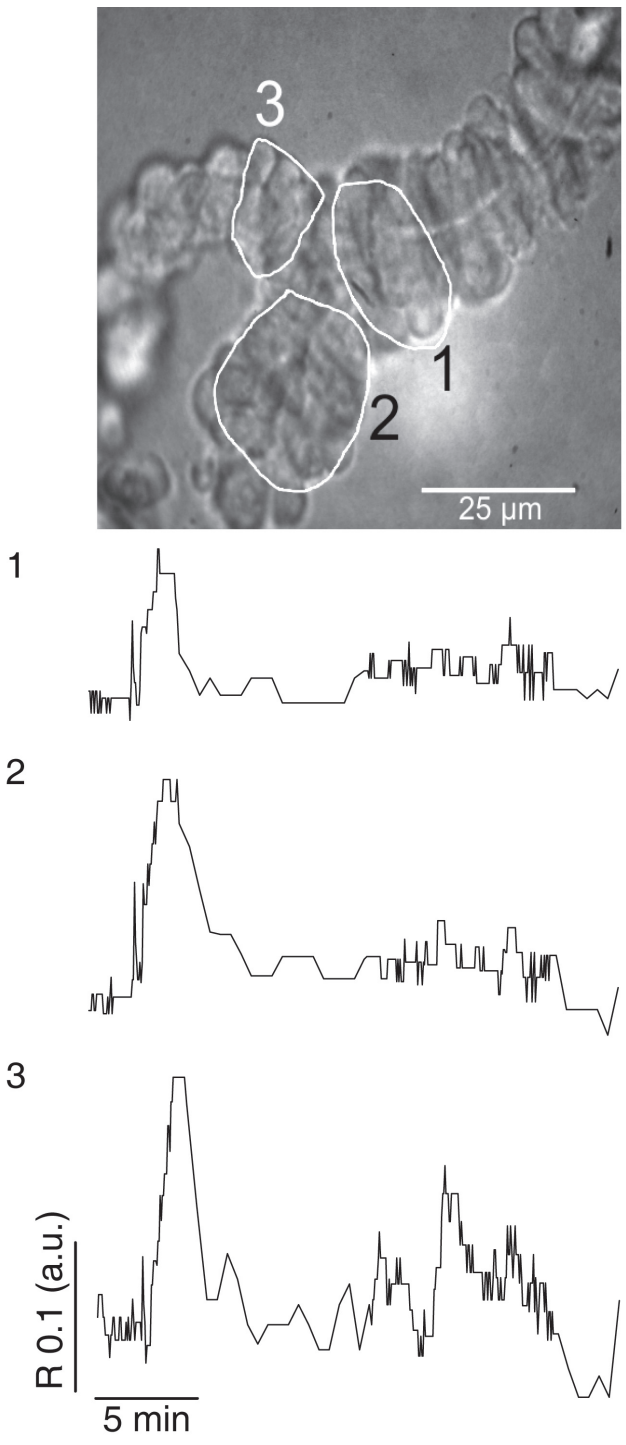

Figure 4. Localisation of the cAMP-induced increase in fura-2 fluorescence ratio R. Upper graph: Interlobular artery and branching of an afferent arteriole. Three regions of interest are shown, before (1) and after the branching (2) and at the beginning of the afferent arteriole (3). The traces show that the three regions responded to superfusion with ANG II (3 nmol/l, 1 min) but only the afferent arteriole (3) responded to stimulation by ISO + IBMX with a increase in R.

analogue, elicit an increase in $[Ca^{2+}]_i$. The response had an all-or-nothing characteristic, i.e. it did not occur always; but when it occurred, it had about the same size (~35% of the response to ANG II (3 nmol/l)). Augmenting the stimulus increased the number of responding organs to up to 90%. cAMP levels were not determined here; however, experiments in isolated renin-secreting cells have shown that stimulation with ISO, IBMX, forskolin or forskolin + trequensin (a specific PDE III inhibitor) increases the resting level of cAMP about 2–10 times (Kurtz 1986; Friis et al. 2002; Grünberger et al. 2006; Ortiz-Capisano et al. 2007a), i.e. to a reasonable physiological degree.

Mechanistic considerations

The cAMP-induced Ca^{2+} transient could arise by triggering either Ca^{2+} release from intracellular stores or Ca^{2+} influx from the extracellular space or both. In order to distinguish between these possibilities, three approaches were used. First, Ca^{2+} was removed from the bath to prevent Ca^{2+} influx. This manoeuvre reduced the calcium peak by ~90% (Tab. 1) showing that the transient arises predominantly from Ca^{2+} influx. Second, inhibitors of sarcolemmal Ca^{2+} channels were used. There has been several publications showing the involvement of L-type Ca^{2+} channels in the ANG II-induced increase in $[Ca^{2+}]_i$ (Kornfeld et al. 1997; Loutzenhiser and Loutzenhiser 2000; Purdy and Arendshorst 2001). However, the dihydropyridine Ca^{2+} channel blocker, isradipine at 100 nmol/l, a concentration which abolished the increase in $[Ca^{2+}]_i$ generated by cell-depolarisation with 60 mmol/l K^+ (Russ et al. 1999), left the cAMP-induced Ca^{2+} unchanged. Hence, a contribution of L-type Ca^{2+} channels to the cAMP-induced Ca^{2+} influx can be ruled out. In addition, stimulation by isoproterenol + IBMX left membrane potential unaltered at approx. –60 mV (see also Bührle et al. 1986) but is at variance with the hyperpolarisation observed by Fishman (1976) and Friis et al. (2003).

The lack of effect on membrane potential renders the activation of other voltage-operated Ca^{2+} channels unlikely. However, the SOCC inhibitors, 2-APB (30 µmol/l) and Gd^{3+} (50 µmol/l) abolished the transient (but left the major part of the response to ANG II). Despite the limited selectivity of these compounds for SOCCs (Parekh and Putney 2005), these results may be taken as first evidence for store-operated Ca^{2+} entry as the major mechanism underlying the transient. Third, the effect of the inhibitors of the SERCA Ca^{2+} pump, thapsigargin and CPA, was investigated. Preincubation with thapsigargin or CPA prevented or greatly reduced (CPA) the Ca^{2+} response to cAMP elicited in the presence of the inhibitor (Tab. 1). How can one understand this result? These agents deplete Ca^{2+} stores (see Ca^{2+} increase in Fig. 3). Thereby they activate store-operated Ca^{2+} entry maximally or greatly (Parekh and Putney 2005); hence

further stimulation of the process by cAMP is either ineffective (thapsigargin) or only slightly effective (CPA). For the validity of this interpretation it is important to note that cAMP-induced Ca^{2+} entry is not inactivated by an increase in $[Ca^{2+}]_i$ (Tab. 1). Furthermore, Ca^{2+} entry induced by forskolin + IBMX is maintained for 10 min or longer (not shown), i.e. the process does not inactivate with time.

Some effort was also made to elucidate the signalling chain leading from the increase in cAMP to Ca^{2+} influx. The response was not affected by the PKA inhibitors H89, and KT 5720 ruling out signalling *via* activation of PKA. The inability of the specific EPAC activator, 8-pCPT-2'-O-Me-cAMP, to elicit the response shows that EPACs are not involved. The possibility that an increase in cAMP may induce Ca^{2+}-mobilization from intracellular stores thereby activating SOCCs is highly unlikely for two reasons: First, Ca^{2+} depletion reduces the response to ANG II (which depends on both Ca^{2+} influx and Ca^{2+} mobilisation from stores; Kurtz and Penner 1989) by only ~50%, suggesting that this manoeuvre leaves Ca^{2+} release from stores largely intact. The response to cAMP was, however, reduced by >90%; the small Ca^{2+} signal remaining in Ca^{2+}-free medium (Fig. 3a; Tab. 1) might originate from Ca^{2+} ions still complexed in the glycocalyx and collagen fibrils of our freshly isolated preparation or may be of a completely different origin. Second, the SOCC inhibitors Gd^{3+} and 2-APB left the response to ANG II largely intact but abolished that to cAMP.

Collectively, the results suggest that cAMP activates a Ca^{2+} influx by a direct interaction with an unknown target. The properties of the cAMP-induced Ca^{2+} entry are neither compatible with a direct activation of cyclic nucleotide-gated channels nor of ion channels of the transient receptor potential superfamily since the former are not sensitive to 2-APB or Gd^{3+} at the concentrations used here (Bradley et al. 2005) and the latter are not activated by cAMP (Clapham et al. 2005).

Potential physiological relevance

We have found here that the cAMP-induced Ca^{2+} increase occurred in the rat afferent arteriole only but not in interlobular arteries. Furthermore, the effect of cAMP on the resting Ca^{2+} level was neither observed in JGC from mice (Kurtz and Penner 1989) nor in afferent arterioles from rabbits (Kornfeld et al. 2000), although cAMP reduced the Ca^{2+} response to stimulation by ANG II (Kurtz and Penner 1989) or noradrenaline (Kornfeld et al. 2000). Could there be any physiological relevance to the peculiar cAMP effect on $[Ca^{2+}]_i$ reported here? First, we recall that under our conditions (afferent arterioles from NaCl-depleted rats, region of interest near to the vessel pole) the cells contributing to the signal are mostly renin-secreting cells (Hackenthal et al. 1990; Leichtle et al. 2004). Keeping in mind that cAMP is the major stimulus of renin secretion (Chen et al. 2007) and that increase in $[Ca^{2+}]_i$ generally decrease the rate of renin secretion (Schweda and Kurtz 2004) one might speculate that the cAMP-induced increase in $[Ca^{2+}]_i$ observed here in rat JGC is a negative feed-back mechanism that limits and fine-tunes the activating effect of cAMP on the activity of the RAAS.

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