Dual mechanism for cAMP-dependent modulation of Ca^{2+} signalling in articular chondrocytes

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The ability of cAMP to modulate the actions of Ca^{2+} -mobilizing agonists was studied in single Fura-2-loaded pig articular chondrocytes in primary culture. Forskolin and 8-Br-cAMP increased both the frequency and amplitude of Ca^{2+} oscillations induced by ATP, and, in unstimulated cells, induced single Ca^{2+} transients or even Ca^{2+} oscillations. The cAMP-dependent protein kinase inhibitor H89 totally prevented the effect of cAMP-elevating agents on Ca^{2+} signalling. Forskolin and 8-Br-cAMP promptly increased the rate of Mn^{2+} quenching, when administered in the presence of ATP, suggesting a potentiation

of receptor-mediated Ca^{2+} influx. In Ca^{2+} -free medium, ATP-induced Ca^{2+} oscillations decreased and stopped after a few cycles: subsequent ATP additions temporarily resumed the activity, an effect that could be mimicked by forskolin. The same agent induced single Ca^{2+} transients in 42 % of the cell population maintained in Ca^{2+} -free medium. Thapsigargin prevented Ca^{2+} responses to both ATP and forskolin. The results indicate a dual mechanism for cAMP-induced potentiation of Ca^{2+} signalling in articular chondrocytes: an increase of receptor-mediated Ca^{2+} influx and a positive modulation of intracellular Ca^{2+} release.

INTRODUCTION

Intracellular Ca^{2+} and cAMP, as intracellular second messengers involved in transduction of extracellular signals, play a major role in the regulation of cell metabolism [1,2]. In chondrocytes from articular cartilage, either of two signalling pathways can be activated by agents normally present in synovial fluid [3], while a wide array of stimulants is known to reach cartilage cells under pathological conditions [4–6].

Cross-talk between Ca^{2+} and cAMP, at the level of signal transduction, is known to occur in several cell types with different resulting outputs: in neutrophils and platelets an inhibitory action of cAMP on Ca^{2+} -mediated pathways has been described [7,8], while in hepatocytes, pancreatic β -cells and FRTL-5 thyroid cells, evidence for cAMP-induced potentiation of Ca^{2+} signalling has been reported [9–13].

Cytosolic Ca^{2+} oscillations, deriving from the rhythmic discharge of Ca^{2+} from intracellular stores, represent a widespread form of signal transduction induced, in many cell types, by receptor-mediated polyphosphoinositide hydrolysis [1]. In some cell systems, elevated levels of cAMP are known to modulate Ca^{2+} oscillations by increasing both their frequency and amplitude, or even by inducing them in cells stimulated with subthreshold doses of InsP_3 -increasing agonists [9–11]. In these cases signal potentiation was derived either from stimulation of Ca^{2+} influx [10], or from cAMP-dependent sensitization of intracellular Ca^{2+} release [9,11].

Extracellular ATP, that is present at elevated levels in the synovial fluid of arthritic patients [14] and stimulates cartilage matrix degradation [15], induces long-lasting Ca^{2+} oscillations in cultured articular chondrocytes [16] by acting on P_{2u} purinoceptors [17].

Present data, obtained using a videoimaging technique on Fura-2-loaded cells, demonstrate that, in articular chondrocytes, a potentiation of Ca^{2+} signalling can be induced by cAMP-

dependent phosphorylation of crucial intracellular targets, and provide evidence for its mechanism. cAMP-elevating agents, such as forskolin (FK) and 8-Br-cAMP, not only increase oscillation frequency and amplitude in ATP-stimulated cells, but even induce Ca^{2+} transients and oscillations in unstimulated chondrocytes. In cells bathed in normal extracellular Ca^{2+} , cAMP increases receptor-mediated Ca^{2+} influx in ATP-stimulated, but not in unstimulated, cells. In cells maintained in Ca^{2+} -free medium, cAMP induces single Ca^{2+} transients in unstimulated cells, while, in cells pretreated with low concentrations of ATP, it induces a long-lasting oscillatory activity. Cell responses to cAMP-elevating agents are totally abolished by thapsigargin (Tg), demonstrating the involvement of intracellular Ca^{2+} stores. In conclusion it is proposed that cAMP may potentiate Ca^{2+} signalling by acting both on receptor-mediated Ca^{2+} influx and on intracellular Ca^{2+} release.

MATERIALS AND METHODS

Cell cultures

Articular cartilage was obtained from the scapula-humerus joints of 150–200 kg pigs killed in an abattoir. The tissue was rinsed in PBS (in mM: 137 NaCl, 2.7 KCl, 8.1 Na_2PO_4 , 1.5 KH_2PO_4 , 0.9 CaCl_2 , 0.49 MgCl_2 ; pH 7.4) and minced into small pieces. The tissue was dissociated by treatment with 250 units/ml collagenase (CLC type II, Worthington, U.S.A.) in the presence of penicillin (500 units/ml) and streptomycin (500 $\mu\text{g}/\text{ml}$). The digestion proceeded at 37 °C for 14–18 h under mild agitation. At the end of this period, the suspension was filtered and centrifuged for 10 min at 700 g. Chondrocytes were plated at a density of 5×10^5 cells/ cm^2 on to polyornithine (10 $\mu\text{g}/\text{ml}$, Sigma)-coated coverslips in Dulbecco's Minimal Essential Medium supplemented with 10 % (v/v) fetal-calf serum and 2 mM

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; FK, forskolin; Tg, thapsigargin.

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L-glutamine, and cultured at 37 °C in a humidified atmosphere containing 5 % CO₂ for 3–6 days, until semiconfluent monolayers of 20–30 cells were observed.

Ca²⁺ imaging

Articular chondrocytes were loaded at room temperature with Fura-2 (3 μ M), dissolved in Pluronic 20 % (1:2, w/v) and added to a solution containing (in mmol/l): NaCl, 125; KCl, 5; MgSO₄, 1; KH₂PO₄, 0.7; CaCl₂, 2; glucose, 6; Hepes-NaOH buffer, 25 (pH 7.4); and 1 % BSA (fraction V). After 40 min the loading solution was removed and the cells washed three times with the same solution (without Fura-2 and BSA). Videomicroscopy and Ca²⁺ measurements were carried out at 37 °C. Drugs were dissolved in the incubation medium and directly perfused in the proximity (50–100 μ m) of the cells. The digital fluorescence-imaging microscopy system is built around a Zeiss inverted Axiovert 100 TV microscope. Cells were excited at 340 and 380 nm (or 360 nm) by a modified Jasco CAM-230 dual-wavelength microfluorimeter. Fluorescence images, collected through a Zeiss achrostat $\times 40$ 1.8 NA oil-immersed objective, were captured by a low-light-level CCD camera (Hamamatsu Photonics) and fed into a digital-image processor developed in the laboratory. Video frames were then digitized, integrated and processed off-line to convert fluorescence data into Ca²⁺ maps (340/380 nm excitation wavelength ratio method). Calibration of Fura-2 fluorescence in terms of intracellular Ca²⁺ concentration ([Ca²⁺]_i) was calculated from the ratio of 340/380 nm fluorescence values (after subtraction of background fluorescence) according to the method of Grynkiewicz et al. [18].

Ionomycin (40 μ M) and extracellular solutions of high Ca²⁺ (10 mM) and low Ca²⁺ (10 mM EGTA–Tris, pH 8) concentration were applied to ascertain the fluorescence ratios in the presence of saturating Ca²⁺ ($R_{\max}$), in the absence of Ca²⁺ ($R_{\min}$) and the B value. Mean values in discrete areas of interest were calculated from sequences of images. Quantitative temporal analyses in spatially restricted areas were thus obtained throughout the experiment.

RESULTS

Stimulation of articular chondrocytes with extracellular ATP (10–500 μ M) induced repetitive Ca²⁺ spikes in 72 % of the cells tested (166 out of 240 cells). Starting from a resting level of 81 ± 9 nM, the [Ca²⁺]_i rhythmically increased to 308 ± 101 nM ($n = 166$). Each Ca²⁺ spike was characterized by a rapid onset (2–6 s), followed by a decline to resting levels. As previously observed [16], in 30 % of the cell population ATP induced only a single, transient, Ca²⁺ increase (results not shown).

To assess the influence of cAMP on ATP-induced Ca²⁺ signalling, cells were treated either with the diterpene FK, which is known to rapidly increase intracellular cAMP by directly activating adenylate cyclase [19], or with the membrane-permeant cAMP analogue 8-Br-cAMP.

Treatment with FK (20 μ M) of oscillating cells prestimulated with low ATP concentrations (10–50 μ M) consistently led to the potentiation of the oscillatory activity, with an increase of both oscillation frequency and amplitude (388 ± 79 nM, $n = 54$, Figure 1A), while, in less responsive cells, FK induced Ca²⁺ oscillations ($n = 21$, Figure 1B). Increasing the concentration of either ATP (100–500 μ M) or forskolin (40–100 μ M) consistently gave rise to an elevated and sustained Ca²⁺ response (results not shown). Similar results were obtained with the membrane-permeant cAMP analogue 8-Br-cAMP (500 μ M, $n = 12$, Figure 1C). When the same protocols were applied on chondrocytes preincubated

Figure 1 cAMP potentiates ATP-induced Ca²⁺ signalling

Representative tracings of Ca²⁺ responses in cells maintained in physiological external Ca²⁺ (2 mM). (A) FK (20 μ M) increases both amplitude and frequency of ATP-induced Ca²⁺ oscillations (ATP 10 μ M). (B) FK (20 μ M) induces Ca²⁺ oscillations in cells where ATP (40 μ M, final concentration) fails to elicit rhythmic activity. (C) 8-Br-cAMP (500 μ M) induces Ca²⁺ oscillations in cells pretreated with low ATP concentrations (10 μ M). (D) Cells preincubated with the inhibitor of cAMP-dependent protein kinase, H-89 (20 μ M, 30 min), can be induced to oscillate by ATP (30 μ M), but fail to show any response to FK (20 μ M). In this particular case FK was even effective in decreasing the oscillation frequency, a result observed in 10 % of the cells tested.

with the inhibitor of cAMP-dependent protein kinase, H-89 (20 μ M, 30 min) [20], we failed to detect any effect of FK or 8-Br-cAMP on Ca²⁺ oscillations ($n = 14$, Figure 1D), suggesting that potentiation occurred through cAMP-mediated phosphorylation.

The possibility that FK could affect Ca²⁺ homeostasis independently of the presence of InsP₃-increasing agonists was evaluated. When resting cells were directly challenged with FK (20 μ M), an increase of cytosolic Ca²⁺ was recorded in a fraction of the cell population (19 out of 47 cells). Such a response could be represented either by a single Ca²⁺ transient ($n = 11$, Figure 2A), or by a train of irregular Ca²⁺ oscillations ($n = 8$, Figure 2B). In both cases the onset of Ca²⁺ transients was significantly slower than those induced by ATP. Under these conditions, subsequent ATP administration (10–30 μ M) con-

Figure 2 FK induces Ca²⁺ responses in unstimulated cells

Representative tracings of Ca²⁺ responses in cells maintained in physiological external Ca²⁺ (2 mM). (A) FK (20 μ M) induces a single Ca²⁺ transient in silent cells. Subsequent stimulation with ATP (10 μ M) elicits Ca²⁺ oscillations with elevated frequency and amplitude. (B) FK (20 μ M) induces irregular Ca²⁺ oscillations in silent cells. ATP (10 μ M) significantly increases both the frequency and the amplitude of the Ca²⁺ spikes.

Figure 3 FK increases ATP-stimulated Ca^{2+} influx

Manganese quenching experiments illustrating the effect of FK on Ca^{2+} influx. The 380-nm tracings show the Ca^{2+} responses, while the 360-nm tracings show the rate of fluorescence quenching induced by Mn^{2+} . (A) The basal rate of quenching, assessed by extracellular addition of Mn^{2+} , that increases after ATP ($30 \mu\text{M}$) administration, is further enhanced by FK ($20 \mu\text{M}$), which induces Ca^{2+} oscillations. (B) The same result is observed when FK ($20 \mu\text{M}$) is added after a maximal ATP administration ($500 \mu\text{M}$). (C) In the absence of ATP stimulation, FK ($20 \mu\text{M}$) does not affect the basal rate of fluorescence quenching, which increases only after ATP ($30 \mu\text{M}$) administration.

sistently stimulated a regular and sustained Ca^{2+} rhythmic activity.

Ca^{2+} oscillations are known to be stimulated both by InsP_3 and by cytosolic Ca^{2+} [1]. Given the modulatory role of cAMP-mediated phosphorylation on ion channels controlling Ca^{2+} influx at the plasma membrane [10], the possibility was evaluated that cAMP could potentiate the Ca^{2+} rhythmic activity by increasing Ca^{2+} influx. The Mn^{2+} -quenching technique was employed to directly assess Ca^{2+} influx: manganese penetrates into the cells through the widespread family of voltage-insensitive Ca^{2+} channels and, by binding to Fura-2 inside the cells, leads to fluorescence quenching [21]. By simultaneously recording the Ca^{2+} signal (at 380 nm) and the Fura-2 quenching (at 360 nm) it is possible to relate the Ca^{2+} increase to Ca^{2+} influx. Mn^{2+} ($100 \mu\text{M}$) was added to the extracellular solution at the beginning of the experiment, to evaluate the rate of basal quenching; in a first series of experiments, ATP (10 – $50 \mu\text{M}$) was subsequently added to induce Ca^{2+} oscillations ($n = 15$, Figure 3A) and FK ($20 \mu\text{M}$) was finally administered, to potentiate the activity.

As already observed [16], ATP increased the rate of Mn^{2+} quenching. Under these conditions FK, besides stimulating Ca^{2+} oscillations, further increased the Mn^{2+} quenching rate, suggesting a cAMP-dependent potentiation of Ca^{2+} influx. The same potentiation was observed when FK ($20 \mu\text{M}$) was administered after the cells had been challenged with a maximal dose of ATP ($500 \mu\text{M}$, Figure 3B). When, on the other hand, FK ($20 \mu\text{M}$) was administered to unstimulated cells, it consistently failed to affect the rate of Mn^{2+} quenching ($n = 18$, Figure 3C), suggesting that cAMP increases Ca^{2+} influx through ATP-stimulated pathways. Similar results were obtained with 8-Br-cAMP (results not shown).

The role of intracellular Ca^{2+} stores in triggering and sustaining the oscillatory activity in non-excitable cells is well documented

Figure 4 cAMP stimulates Tg-sensitive intracellular Ca^{2+} stores

Representative tracings of Ca^{2+} responses in cells bathed in Ca^{2+} -free, EGTA-containing medium (EGTA 0.5 mM). (A) Oscillations induced by ATP ($50 \mu\text{M}$) decrease and eventually stop. Stepwise increases of ATP concentration (100 , 300 , $700 \mu\text{M}$) induce one or more Ca^{2+} spikes. A final challenge with ATP (1 mM) failed to elicit any signal, suggesting depletion of intracellular store content. (B) FK ($20 \mu\text{M}$) resumes the oscillatory activity induced by ATP ($30 \mu\text{M}$). (C) FK ($20 \mu\text{M}$) induces a single Ca^{2+} transient in unstimulated cells and increases cell sensitivity to low ATP concentrations ($10 \mu\text{M}$). After exhaustion of the oscillatory activity, treatment with Tg ($1 \mu\text{M}$) induces a single Ca^{2+} transient. (D) Tg ($1 \mu\text{M}$) induces Ca^{2+} transients and prevents a cell response to subsequent ATP ($50 \mu\text{M}$) and FK ($20 \mu\text{M}$) administration.

[1]. Similarly, the role of cAMP in modulating the activity of intracellular stores has been reported [9,11]. Given the potentiating effect of cAMP on Ca^{2+} signalling, the possibility was evaluated that cAMP, besides increasing Ca^{2+} influx, could modulate intracellular Ca^{2+} release in articular chondrocytes.

We have previously demonstrated that Ca^{2+} oscillations could be induced in articular chondrocytes bathed in Ca^{2+} -free media: under these circumstances, however, the rhythmic activity decreased with time, until Ca^{2+} spikes were no longer detectable [16]. The possibility that, under these conditions, the exhaustion of the activity resulted from depletion of intracellular Ca^{2+} stores was investigated. Cells, maintained in Ca^{2+} -free, EGTA-containing medium, were challenged with increasing concentrations of ATP ($n = 8$, Figure 4A). As already described, the oscillatory activity induced by low ATP concentrations ($30 \mu\text{M}$) decreased progressively and disappeared. Subsequent stepwise increases of agonist dose (up to 1 mM) were revealed to be effective in inducing single Ca^{2+} transients or repetitive Ca^{2+} spikes, suggesting that the exhaustion of oscillations resulted from a diminished intracellular store responsiveness.

The effect of cAMP on intracellular Ca^{2+} release was evaluated in cells bathed in Ca^{2+} -free, EGTA-containing medium. In an initial series of experiments oscillations were induced with ATP (30 – $50 \mu\text{M}$); after exhaustion of the activity, treatment with FK ($20 \mu\text{M}$) promptly restored it, with oscillations maintained for up to 15 min ($n = 12$, Figure 4B). When FK (20 – $200 \mu\text{M}$) was administered to unstimulated cells, a single Ca^{2+} transient could be recorded (26 out of 40 cells, Figure 4C). In the presence of FK, addition of a low ATP concentration ($10 \mu\text{M}$) consistently evoked Ca^{2+} rhythmic activity that decreased with time and eventually

stopped; under these conditions, Tg (1 μ M) could still induce a Ca^{2+} transient, thus demonstrating that the progressive inhibition of the oscillatory activity was not due to depletion of the intracellular store content. Tg, in fact, leads to Ca^{2+} stores discharge by inhibiting the Ca^{2+} -ATPase responsible for intracellular store refilling [22]. When Tg (1 μ M) was administered to unstimulated cells it induced a single Ca^{2+} transient and completely abolished the response elicited by both ATP and FK ($n = 7$, Figure 4D), thus demonstrating that ATP and FK elicited their effects by acting on the same Tg-sensitive intracellular Ca^{2+} stores.

DISCUSSION

Cytosolic Ca^{2+} and cAMP both act as pleiotropic second messengers in cartilage cells [3]. Extracellular ATP, acting through P_2 purinoreceptors [17], is known to induce Ca^{2+} oscillations in cultured articular chondrocytes [16]. Present results demonstrate that the interaction between the two second messengers into converging signalling pathways gives rise to a potentiation of the agonist-induced Ca^{2+} signalling, resulting in increased frequency and amplitude of the Ca^{2+} oscillatory activity, or even in the induction of Ca^{2+} oscillations in silent, unstimulated cells. In this study we explored the mechanism responsible for this potentiation.

Ca^{2+} oscillations, particularly their frequency, are known to be related to the extent of cell stimulation, thus representing a digital form of intracellular signal encoding [1]. In the different oscillating systems, frequency is linked either to the concentration of the polyphosphoinositide-mobilizing agonist(s), or to the extent of Ca^{2+} influx. In both cases the rhythmic activity results from the repetitive discharge of Ca^{2+} from specialized intracellular compartments, and is mediated by the binding of $\text{Ins}(1,4,5)\text{P}_3$ to its receptor, an intracellular Ca^{2+} -releasing channel [1]. In the presence of the ligand, the activity of the InsP_3 receptor can be positively modulated, at its cytosolic face, by several intracellular metabolites, among which are Ca^{2+} and cAMP [23,24], while, at the intraluminal side, it appears to be controlled by the extent of Ca^{2+} store loading [25]. In all cases, the positive modulation is expected to increase the receptor affinity for its ligand [23–25].

In articular chondrocytes, cAMP-evoked potentiation of Ca^{2+} signalling, mediated by cAMP-dependent phosphorylation of intracellular constituents, occurs through a dual mechanism, involving both an increased Ca^{2+} influx and a stimulated intracellular Ca^{2+} release.

Regarding the first mechanism, the influence of cAMP-mediated phosphorylation on different Ca^{2+} influx pathways is reported for several cell systems; in hypersecreting pancreatic β -cells from obese mice, cAMP is known to induce Ca^{2+} oscillations by increasing Ca^{2+} influx through voltage-dependent pathways [12], while in hepatocytes cAMP-dependent phosphorylation stimulates receptor-mediated Ca^{2+} entry [10]. In other systems, however, cAMP is known to inhibit Ca^{2+} influx, thus antagonizing overall Ca^{2+} signalling [26]. In articular chondrocytes FK increased the rate of manganese quenching in ATP-stimulated, but not in unstimulated, cells, suggesting a positive modulation of an agonist-stimulated entry mechanism. Regarding the influx pathway involved, a role for receptor (second messenger)-mediated Ca^{2+} entry seems likely [27]. In fact, the ability of FK to increase the manganese quenching rate after the cells had been challenged with a maximal dose of ATP (which is expected to mobilize all the intracellular Ca^{2+}) rules out the possible involvement of the pool-controlled influx pathway (I_{CRAC}), which does not seem to be active in these cells [16].

The ability of FK to resume ATP-induced oscillatory activity exhausted under Ca^{2+} -free conditions, closely resembled the effect of increased agonist doses, and could be related to the intraluminal control by Ca^{2+} of InsP_3 receptor activity. It is, in fact, predicted that in the absence of extracellular Ca^{2+} the intracellular stores become progressively discharged of their content upon repetitive stimulation: this would reduce the responsiveness of the InsP_3 receptor, that would require higher InsP_3 concentrations to become activated [25]. Under these conditions, a positive modulation of receptor activity at its cytosolic face could overcome the loss of sensitivity. It is worth noting, in this regard, that cAMP-dependent phosphorylation is known to increase both the rate and the extent of Ca^{2+} fluxes through the purified type-I InsP_3 receptor [24], and that, in permeabilized hepatocytes, cAMP potentially enhanced InsP_3 -induced Ca^{2+} release [9].

The fact that FK could induce single Ca^{2+} transients in unstimulated chondrocytes bathed in Ca^{2+} -free medium, and Ca^{2+} oscillations in cells maintained in physiological extracellular Ca^{2+} , suggests a cAMP-dependent positive modulation of intracellular Ca^{2+} discharge. Although the possibility that increased cAMP levels could stimulate InsP_3 synthesis cannot be ruled out, a stimulatory role of cAMP-dependent phosphorylation of InsP_3 receptor appears more likely. In fact, in contrast to ATP, FK (even at high concentrations) consistently failed to induce Ca^{2+} oscillations in Ca^{2+} -free medium, suggesting a distinct mechanism of action from InsP_3 -generating agonists.

cAMP-mediated stimulation of Ca^{2+} signalling is likely to have significant consequences for chondrocyte physiology and pathology: for agonists like parathyroid hormone or histamine, that are known to induce cAMP and/or InsP_3 synthesis, the cooperativity between the two signalling pathways may enhance overall cell sensitivity and lead to an increased cellular output. Cell metabolism, prostaglandin production and matrix degradation represent some of the cell processes that are known to be stimulated by elevated intracellular Ca^{2+} and cAMP levels [5,15]. The presence of a wide array of cAMP and InsP_3 -stimulating agonists at sites involved in joint diseases is suggestive of massive cellular activation: given the cytotoxic action of elevated Ca^{2+} concentrations, a role might be postulated for a synergistic action of cAMP and Ca^{2+} in the extensive cell death associated with degenerative diseases.

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