Heat treatment induces an increase in intracellular cyclic AMP content in human epidermoid A-431 cells

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The basal level of intracellular cyclic AMP (cAMP_i) in A-431 cells incubated at 37 °C in Na⁺-containing Hanks solution is 2086 ± 139 fmol/10⁶ cells. When cells are exposed to 45 °C for 10 min, cAMP_i increases by $40 \pm 4\%$, and then returns to basal levels within 30 min. Incubating cells in Ca²⁺-free or Mg²⁺-free Hanks solution has no effect on the heat-induced increase in cAMP_i, but the increase is inhibited by acid-loading cells to intracellular pH 7.0 or 6.8. In unheated cells, cAMP_i increases by $16 \pm 8\%$, $53 \pm 7\%$, or $39 \pm 8\%$ when incubated with 3-isobutyl-1-methylxanthine (1 mM), Ro 20-1724 (0.5 mM), or theophylline (1 mM) respectively. However, heat treatment further elevates cAMP_i in cells treated with phosphodiesterase inhibitors, indicating that heat treatment and phosphodiesterase inhibitors elevate cAMP_i by a different pathway(s). Heat treatment increases adenylate cyclase activity 2.5-fold. When forskolin (150 μM), an adenylate cyclase stimulator, is applied to cells, the basal cAMP_i increases 28 ± 6 -fold compared with controls. Subsequent heating of these cells lowers cAMP_i levels to 7.0 ± 0.5 times that in control cells. This decrease is prevented by pretreatment with pertussis toxin (30 ng/ml, 24 h), suggesting that G-proteins are involved in the process of heat-induced cAMP_i increase. 2-Deoxy-D-glucose (10 mM), NaN₃ (10 mM) and 2,4-dinitrophenol (1 mM) also increase cAMP_i in A-431 cells. However, application of these metabolic inhibitors to cells before heat treatment does not result in cAMP_i levels greater than that observed in cells with heat alone. Similar observations are obtained in heat-treated cells previously exposed to adenosine, but not to AMP or ADP. These data are the first to suggest that thermally induced increase in cAMP_i is due to a combination of activation of adenylate cyclase and G-proteins, and an increase in adenosine owing to ATP breakdown caused by hyperthermia.

INTRODUCTION

Heat exposure acts primarily at the plasma membrane, and causes changes in many intracellular parameters [1]. For example, heat induces an intracellular acidification, due partly to an inhibition of the Na⁺/H⁺ antiporter [2,3]. Heat also increases intracellular Ca²⁺ [4,5] by inhibiting Ca²⁺-ATPase activity [5]. Previous reports have demonstrated that intracellular cAMP levels (cAMP_i) are changed with hyperthermia. Kampa & Frascella [6] reported that cAMP levels in rabbit epididymis increase during hyperthermia, and that the increase could be inhibited by exogenous insulin. Lin *et al.* [7] indicated that heating thymocytes for 30 min or longer at 43 °C causes a massive rise in cAMP_i. On the other hand, Ehrlich carcinoma cells subjected to 45 °C demonstrate a decrease in cAMP_i, related perhaps to an increase in intracellular Ca²⁺ [5].

Interactions between intracellular pH (pH_i) and cAMP have been studied. Boron *et al.* [8] observed that 1 μM-cAMP increases pH_i by 0.1 unit in internally dialysed barnacle muscle fibres set to pH_i 6.9. In hypercapnic myocardium, dibutyryl cAMP in millimolar concentration increases pH_i by 0.2 unit [9,10]. However, intracellular acidification produced by exogenous cAMP has also been observed in the normoxic rat liver [11], A-431 cells [12] and renal tubular cells [13]. Although responses differ among cell types, evidence indicates that modulation of pH_i has important effects on cAMP_i. Yajima & Ui [14] assayed cAMP levels in the livers of alkalotic and acidotic rats, and found them to be significantly elevated or depressed, respectively. It has been shown that heat shock alters pH_i in A-431 cells [3]. The mechanism relating changes in pH_i to cAMP_i is not known.

In this study we report that heat shock induces an increase in cAMP_i in A-431 cells. We have characterized the relationship between temperature and cAMP_i and investigated the underlying mechanisms of changes in cAMP_i that occur following heat shock. A preliminary account of these findings has been presented [15].

MATERIALS AND METHODS

Cells

Human epidermoid carcinoma A-431 cells (American Type Culture Collection, Rockville, MD, U.S.A.) were grown in 75 cm² tissue-culture flasks (Costar, Cambridge, MA, U.S.A.) containing Dulbecco's Modified Eagle medium with 0.03 % glutamine, 4.5 g of glucose/l, 24 mM-Hepes, 10 % (v/v) fetal bovine serum, penicillin (50 μg/ml) and streptomycin (50 units/ml) (Gibco, Grand Island, NY, U.S.A.), and incubated at 37 °C in a humidified atmosphere of 5 % CO₂ in air. Cells were fed every 3–4 days. Cells from passages 28–50 were used for experiments.

Heat treatment

Confluent monolayers of cells (final cell density 2×10^6 cells per 35 mm culture dish) were washed twice with phosphate-buffered saline (Whittaker M.A. Bioproducts, Walkersville, MD, U.S.A.) and incubated with phosphate-buffered saline for 1 h at 37 °C. Cells were then incubated with 3-isobutyl-1-methylxanthine (IBMX; final concn. 1 mM) for 30 min at 37 °C. After the incubation with IBMX, the medium was removed. Heat treat-

Abbreviations used: cAMP_i, intracellular cyclic AMP; pH_i, intracellular pH; IBMX, 3-isobutyl-1-methylxanthine; p[NH]ppG, guanosine 5'-[βγ-imido]triphosphate; 2-DG, 2-deoxy-D-glucose; DNP, 2,4-dinitrophenol.

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ment was conducted by exposing cell monolayers to Na⁺-containing Hanks solution at 37, 40, 45 or 50 °C and placing the cells in an incubator at the same temperature for a specified period of time. The temperatures and duration of heat treatment were chosen on the basis of our previous experiments [3]. The medium was discarded, and 1 ml of 0.1 M-HCl was added to each culture dish to stop the reaction by killing the cells. The culture dishes were maintained at 4 °C for 1–2 h and then transferred to test tubes. Samples were boiled for 10 min and centrifuged at 2000 *g* for 10 min. The supernatants were kept and stored at –20 °C until cAMP was determined. To measure adenylate cyclase activity, cells were scraped after heat treatment, homogenized, and centrifuged at 12000 *g* for 20 min at 0 °C to collect the cell membranes for the assay.

In order to study the effect of heat on cells with different pH_i, cells were incubated in NH₄Cl-containing (40 mM) Hanks solution for 5 or 8 min, then incubated in Na⁺-free Hanks medium for 2 min to set pH_i at 7.0 and 6.8 respectively.

pH_i measurements

Confluent epithelial cells were placed in Na⁺-containing Hanks solution supplemented with 5 mM-glucose, 0.2 % BSA and 2 μM-2,7-bis(carboxyethyl)carboxyfluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, OR, U.S.A.), and incubated for 30 min at 37 °C. At the end of the incubation period, cells were washed twice with Na⁺-containing Hanks solution. The method for determination of pH_i has been described previously [3]. Briefly, the fluorescence signal was measured on a PTI DeltaScan spectrofluorimeter (Photon Technology International, South Brunswick, NJ, U.S.A.) as the ratio of emission at 527 nm for dual excitation at 500 and 434 nm (slit width 4 nm). BCECF leaked out of A-431 cells at a rate of 0.77 ± 0.26 %/min (*n* = 3), and extracellular dye carried over from washes was 6 ± 1 % (*n* = 3). The fluorescence signal was calibrated by a method modified from that of Thomas *et al.* [16]. Cells were placed in high-K⁺ (145 mM) Hanks solution, nigericin (3 μM) and valinomycin (3 μM) were added, and the cells were incubated for 5 min. The buffer was then titrated with KOH or HCl and the fluorescence signal recorded. The data were fitted by a sigmoid line, and pH_i was calculated by using the fitted parameter values (derived from RS/1 computer software).

cAMP and adenylate cyclase measurements

cAMP was determined by radioimmunoassay by the method of Lin *et al.* [17]. Each sample was measured in duplicate or triplicate. Adenylate cyclase activity was assayed by measuring [³²P]cAMP formed from an [α-³²P]ATP substrate. [³²P]cAMP was purified by sequential chromatography on columns of Dowex cation-exchange resin and aluminium oxide [18]. Radioactivity was measured with a scintillation counter. The reaction was initiated by the addition of membranes to give 20–50 μg of membrane protein/ml in a final reaction volume of 100 μl. The control and heat-shocked membranes were incubated at 30 °C for 5 min. The amount of protein was determined by the micro-Bio-Rad protein assay.

ATP measurements

Cellular ATP was extracted by incubating 1 × 10⁶ cells/sample with ATP-releasing reagent (a NaOH-buffered detergent that disrupts cells; Assay Kit FL-SAC, Sigma Chemical Co., St. Louis, MO, U.S.A.) for 15 min at 0 °C. ATP was assayed by using the luciferase–luciferin assay and a luminometer (Picolite model 6500).

Solutions

Na⁺-containing Hanks solution contained (mM) 148 NaCl,

4.6 KCl, 1.2 MgCl₂, 1.6 CaCl₂, 10 Hepes, pH 7.38. In experiments in which Na⁺-free, Ca²⁺-free or Mg²⁺-free Hanks solution was used, NaCl was replaced by an equimolar concentration of *N*-methyl-D-glucamine, and Ca²⁺ and Mg²⁺ were replaced by equimolar concentrations of EGTA.

Statistical analysis

All data are expressed as means ± S.E.M. Analysis of variance, Bonferroni's inequality, and Student's *t* test were used for comparisons of groups [19].

Chemicals

Ro 20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone] was generously given by Hoffmann-La Roche (Nutley, NJ, U.S.A.). Other chemicals used were adenosine, AMP, ADP, cholera toxin, IBMX, guanosine 5'-[βγ-imido]triphosphate (p[NH]ppG), theophylline, forskolin, 2-deoxy-D-glucose (2-DG), NaN₃, 2,4-dinitrophenol (DNP) and pertussis toxin (Sigma). [¹²⁵I]-cAMP and [α-³²P]ATP were purchased from New England Nuclear (Boston, MA, U.S.A.).

RESULTS

Effect of heat on cAMP_i

The basal cAMP_i in A-431 cells at 37 °C in Na⁺-containing Hanks solution is 2086 ± 139 fmol/10⁶ cells (*n* = 56). Heating A-431 cells to 40–50 °C elevates cAMP_i. As depicted in Fig. 1, heat treatment at 40 °C for 10 min increases cAMP_i by 38 ± 7 % (*n* = 6). Higher temperatures do not promote a further increase in cAMP_i. Heat treatment increases cAMP_i in a time-dependent manner. Fig. 2(a) shows that cAMP_i in cells incubated at 45 °C increases with the duration of heating. The maximal increase is observed between 10 and 20 min; at 30 min, cAMP_i is lower than at 10 and 20 min. This is probably due to a small decrease in viability [90 ± 4 % versus control 100 % (3)]. To determine how long the heated cells maintain the elevated cAMP_i, recovery from an increased level of cAMP_i was examined in cells exposed to 45 °C for 10 min and then returned to 37 °C for times up to 1 h. The data presented in Fig. 2(b) demonstrate that levels of cAMP_i are slightly, but not significantly, higher than the control at 15 and 30 min after the return to 37 °C, and significantly lower than

Fig. 1. Effect of temperature on cAMP_i

Cells were exposed to 37, 40, 45 or 50 °C for 10 min (*n* = 6 for each group). cAMP_i was assayed in triplicate. Data are expressed as percentages of cAMP_i at 37 °C: **P* < 0.05 versus 37 °C.

Fig. 2. Time course of heat-induced increase in cAMP_i

(a) Effect of duration of heat treatment on cAMP_i. Cells were exposed to 37 °C (○) or 45 °C (●) for the indicated time ($n = 6$ for each group). (b) Recovery of cAMP_i after heat treatment. Cells were exposed to 45 °C for 10 min, and then returned to 37 °C for the indicated time (●) (○, control maintained at 37 °C) ($n = 6$ for each group). cAMP_i was assayed in triplicate. Data are expressed as a percentage of cAMP_i at 37 °C at 0 min: * $P < 0.05$ versus 37 °C.

the control at 1 h. The reason for the latter observation is not clear. On the basis of results shown on Fig. 2(a), heat treatment at 45 °C for 10 min was selected for subsequent experiments. Because the basal level of cAMP_i was different in different experiments, each experiment was performed with its own controls.

Because heat treatment increases cytoplasmic Ca²⁺ concentration in A-431 cells to a level 2.5 times that of controls [4], the relationship between cAMP_i and Ca²⁺ was studied. Cells were heated at 45 °C for 10 min in Na⁺-containing Hanks solution without Ca²⁺. The increase in cAMP_i in heated cells is not different from that in cells treated in Ca²⁺-containing Na⁺ Hanks solution (Fig. 3). Similarly, this heat-induced increase in cAMP_i does not depend on the external Mg²⁺ (Fig. 3).

Interactions of cAMP_i and pH_i

Heat treatment has been shown to acidify A-431 cells [2,3]. If

Fig. 3. Heat-induced increase in cAMP_i is independent of external Ca²⁺ or Mg²⁺

Cells were exposed to 45 °C (▨) for 10 min in Ca²⁺-free (EGTA, 10 mM) or Mg²⁺-free (EGTA, 1.3 mM) Hanks medium ($n = 6$ each group). cAMP_i was assayed in triplicate. Data are expressed as percentages of cAMP_i in the control group (which contained both Mg²⁺ and Ca²⁺ at 37 °C): * $P < 0.05$ versus 37 °C (■).

Fig. 4. Intracellular acidification inhibits heat-induced increase in cAMP_i

Cells were acid-loaded by pre-pulsing with NH₄Cl before heat treatment (▨; 45 °C, 10 min) ($n = 6$). cAMP_i was assayed in triplicate. Data are expressed as percentage of cAMP_i at pH_i 7.2 and 37 °C: * $P < 0.05$ versus 37 °C (■); ** $P < 0.05$ versus pH_i 7.2 and 7.0.

this increase in cAMP_i is caused by the heat-induced intracellular acidification, then acid-loading cells should produce an increase in cAMP_i. cAMP_i in unheated cells acid-loaded to pH_i 6.8 or 7.0 remains unchanged (Fig. 4). When cells are exposed to preheated Na⁺-free Hanks solution (45 °C), cAMP_i does not change at pH_i 7.0, but decreases significantly at pH_i 6.8 (Fig. 4).

As an alternative way of testing the relationship between pH_i and cAMP_i, amiloride (100 μM, 10 min) was used to acidify A-431 cells from their resting pH_i of 7.22 ± 0.03 to 7.08 ± 0.01 (3). Amiloride-treated cells at 37 or 45 °C do not exhibit changes in cAMP_i compared with controls (in fmol/10⁶ cells: control, 2679 ± 218 , $n = 11$; amiloride at 37 °C, 2867 ± 193 , $n = 12$; amiloride at 45 °C, 2858 ± 303 , $n = 12$). These experiments suggest that there is no direct relationship between intracellular acidification and the rise in cAMP_i, although pH_i can affect cAMP_i in some cases.

It is possible that heat alters cAMP_i by affecting phosphodiesterase, G-proteins, adenylate cyclase and/or substrates used for cAMP formation. The following experiments were conducted to explore these possibilities.

Effect of heat on phosphodiesterase

Phosphodiesterases hydrolyse cAMP to AMP, and an inhibition of phosphodiesterases leads to an accumulation of cAMP in the cells. The phosphodiesterase inhibitors IBMX, Ro 20-1724 and theophylline [20–22] were used to determine if the

Fig. 5. Pretreatment with phosphodiesterase inhibitors does not prevent heat-induced increase in cAMP_i

Cells were treated with IBMX (1 mM), Ro 20-1724 (0.5 mM) or theophylline (1 mM) for 10 min before heat treatment (▨; 45 °C, 10 min) ($n = 6$). cAMP_i was assayed in triplicate. Data are expressed as percentage of cAMP_i in the control group at 37 °C: * $P < 0.05$ versus 37 °C (■).

Table 1. Effect of pertussis toxin on heat-induced cAMP_i increase in A-431 cells

Cells were treated with pertussis toxin (10 ng/ml, 24 h, 37 °C), and then exposed to forskolin (150 μ M, 10 min) before heat treatment (45 °C, 10 min). Data are expressed as mean percentages of cAMP_i in unheated cells (2057 $\pm$ 171 fmol/10⁶ cells) ($\pm$ S.E.M.; n = 6–44): * P < 0.05 versus unheated control cells; ** P < 0.05 versus unheated and heated control cells (two-way ANOVA and Bonferroni's inequality).

	cAMP _i (%)	
	Unheated	Heated
Control	100	145 $\pm$ 7*
Pertussis toxin	96 $\pm$ 3	200 $\pm$ 16*
Forskolin	2825 $\pm$ 615**	717 $\pm$ 44**
Pertussis toxin + forskolin	4470 $\pm$ 908**	2270 $\pm$ 234**

heat-induced increase in cAMP_i occurs by inhibiting phosphodiesterases. Cells were incubated with IBMX (1 mM), Ro 20-1724 (0.5 mM) or theophylline (1 mM) for 10 min before heat treatment. Their concentrations used here have been shown to produce a maximal inhibition of phosphodiesterases [23,24]. Fig. 5 shows that cAMP_i increases by 16 $\pm$ 4 %, 53 $\pm$ 7 % and 39 $\pm$ 8 % (all n = 6) in unheated cells after the respective treatments, but heating promotes an additional increase in cAMP_i similar to that measured in cells exposed to heat alone, indicating that heat treatment and phosphodiesterase inhibitors stimulate an increase in cAMP_i by different pathways.

It was surprising to us that pretreatment with theophylline (3 mM) plus IBMX (1 mM) for 10 min blocks the heat-induced increase in cAMP_i (37 °C, 2781 $\pm$ 546 fmol/10⁶ cells; 45 °C, 2698 $\pm$ 617 fmol/10⁶ cells; n = 9). This is not observed when the concentration of theophylline is lowered to 1 mM in the presence of IBMX (1 mM) (results not shown). The reason for the inhibition observed with the high concentration is not clear, but is perhaps due to some undefined non-specific activity.

Effect of heat on G-proteins

To determine if heat promotes the interaction between the G-proteins and adenylate cyclase, forskolin, an adenylate cyclase stimulator [25], was applied to cells to maximize adenylate cyclase activity. Cells were incubated with 150 μ M-forskolin (15 mM stock solution in 95 % ethanol) at 37 °C for 10 min [26,27] before cells were exposed to 45 °C for 10 min. Table 1 shows that forskolin itself increases cAMP_i by a factor of 28.25 $\pm$ 6.15. Heat treatment decreases cAMP_i in these forskolin-pretreated cells 7.17 $\pm$ 0.44 times the basal level. To determine if these results indicate that the inhibitory G-protein (G_i) is activated by heat treatment, we measured the effect of heat in the presence of pertussis toxin, a G_i-protein inhibitor. Cells were incubated with pertussis toxin for 24 h. If G_i were activated by heating, treatment of cells with pertussis toxin followed by heat should result in a greater increase in cAMP_i than that observed in cells treated with heat alone. Also, cAMP_i in cells treated with pertussis toxin and forskolin followed by heat should be the same as that in cells treated with forskolin. In the first experiment, pertussis toxin (10 or 30 ng/ml; 24 h, 37 °C) itself did not change the resting level of cAMP_i (Table 1). Exposing pertussis-toxin-treated cells to 45 °C for 10 min increased cAMP_i to a value greater than that in heated cells not exposed to pertussis toxin. In the other experiment forskolin (150 μ M; 10 min) applied to cells treated with pertussis toxin induces an increase in cAMP_i to a

Table 2. Effect of heat treatment on adenylate cyclase activity in A-431 cells

Cells were exposed to 45 °C for 10 min in Na⁺-containing Hanks medium. The adenylate cyclase activity in unheated and heated cells was determined by its ability to convert [α -³²P]ATP into [³²P]cAMP at 30 °C (n = 3): * P < 0.05 versus unheated cells (Student's t test).

	cAMP (pmol/mg of protein)	
	Unheated	Heated
Basal	15.5	39.4*
p[NH]ppG	63.5	141.4*

degree not significantly different from cells not exposed to pertussis toxin. However, the decrease in cAMP_i induced by heat in forskolin-treated cells was attenuated by 10 ng of pertussis toxin/ml and completely prevented by 30 ng of pertussis toxin/ml (results not shown), suggesting that G-proteins are involved.

To corroborate this observation, cholera toxin (1 μ g/ml), an irreversible adenylate cyclase stimulator, was applied 1 h [28] before heat shock to cells pretreated with pertussis toxin (30 ng/ml; 24 h, 37 °C). Like forskolin, cholera toxin alone increases cAMP_i (726 $\pm$ 11 pmol/10⁶ cells; n = 6). Heat treatment attenuates cAMP_i in cholera-toxin-treated cells by 32 $\pm$ 5 % (n = 6). However, pretreatment with pertussis toxin abolishes this heat-induced attenuation of cAMP_i in cholera-toxin-treated cells (668 $\pm$ 33 pmol/10⁶ cells; n = 6).

To determine if G-protein activity recovers from heat treatment, forskolin (150 μ M) was applied to cells 30 min after heat treatment. cAMP_i is 785 $\pm$ 21 pmol/10⁶ (n = 6), indicating that activity of G-proteins recovers.

Effect of heat on adenylate cyclase

Because forskolin treatment increases adenylate cyclase activity, and heat treatment does not increase cAMP_i in cells pretreated with forskolin (Table 1), it seems possible that heat and forskolin increase cAMP_i by the same pathways. To test this possibility, the activity of adenylate cyclase in unheated and heated cells was measured. Table 2 shows that heat treatment increases basal adenylate cyclase activity 2.5-fold. The addition of p[NH]ppG, a non-hydrolysable analogue of GTP, increases the basal activity of adenylate cyclase 4-fold at 37 °C. Heat treatment in the presence of p[NH]ppG increases the activity of adenylate cyclase a further 2.3-fold (Table 2).

Effect of heat on ATP pool

It has been reported that heat inhibits glycolysis, oxidative phosphorylation [3] and cytochrome *c* activity [29], thereby leading to a decrease in ATP contents by about 20 % [3]. It is possible that the decrease in ATP levels is related to the heat-induced increase in cAMP_i. Metabolic inhibitors such as 2-DG, NaN₃ or DNP deplete the ATP pool and subsequently increase ADP, AMP and adenosine levels. To investigate the effect of heat on the ATP pool, cells were treated with metabolic inhibitors and heat. 2-DG (10 mM), NaN₃ (10 mM) or DNP (1 mM) was applied to cells for 30 min, followed by heat treatment at 45 °C for 10 min. Table 3 depicts the ATP content at 37 or 45 °C in cells pretreated with or without metabolic inhibitors. Metabolic inhibitors decrease intracellular ATP content. As shown in Fig. 6, these metabolic inhibitors increase cAMP_i, but heat treatment is not capable of further significantly increasing cAMP_i, even though heat treatment leads to a further decrease in ATP content

Table 3. Effects of metabolic inhibitors and heat on cellular ATP content in A-431 cells

Cells were treated with the specified drug for 30 min and then exposed to 45 °C for 10 min. Values represent means ± s.e.m. (*n* = 3): **P* < 0.05 versus unheated control cells; ***P* < 0.05 versus unheated drug-treated cells (two-way ANOVA and Studentized Range-test).

	ATP (nmol/10 ⁶ cells)	
	Unheated	Heated
Control	3.64 ± 0.07	3.28 ± 0.06*
2-DG	1.81 ± 0.06*	0.06 ± 0.008**
NaN ₃	0.16 ± 0.01*	0.076 ± 0.002**
DNP	0.16 ± 0.01*	0.036 ± 0.005**

(Table 3). Similarly, no further increase in cAMP_i is found if heat is applied before the metabolic inhibitors, suggesting that the increase in cAMP_i by heating or the treatment with metabolic inhibitors shares the same pathways. Metabolic inhibitors do not have effects on adenylate cyclase activity (results not shown).

To determine which metabolite (adenosine, AMP or ADP) is involved in this heat-induced increase in cAMP_i, cells were pretreated with adenosine, AMP or ADP (0.25 mM; [23]) before heating. Table 4 shows that adenosine increases the cAMP_i to 566 ± 5 % in unheated cells, and heat treatment does not further significantly increase cAMP_i. Treatment with ADP increases cAMP_i by 98 ± 19 % in unheated cells, and heating produces an additional increase. This phenomenon is mimicked by adenosine at 0.01 mM and 0.05 mM. Treatment with AMP neither changes the basal level nor prevents the heat-induced increase in cAMP_i.

It is known that the interaction between adenosine and adenylate cyclase can be blocked by methylxanthines in different tissues [30]. When cells were treated with forskolin (150 μM, 10 min) to maximize adenylate cyclase activity, then treated with adenosine (0.25 mM, 30 min), the adenosine-induced increase in cAMP_i is not observed (forskolin, 2470 ± 120 pmol/10⁶ cells, *n* = 3; forskolin + adenosine, 2190 ± 240 pmol/10⁶ cells, *n* = 3). This suggests that adenosine stimulates cAMP_i by increasing adenylate cyclase activity. The combination of adenosine and IBMX (1 mM, 30 min) decreases the effect of adenosine alone on cAMP_i in unheated cells (adenosine, 45 ± 1 pmol/10⁶ cells, *n* = 3; adenosine + IBMX, 16 ± 1 pmol/10⁶ cells, *n* = 3). If the metabolic-inhibitor-induced increase in cAMP_i (Table 3) is due to an elevation in adenosine levels, then a combination of IBMX and a metabolic inhibitor should block the increase in cAMP induced

Table 4. Effect of heat treatment on cAMP_i in cells pretreated with adenosine, AMP or ADP

Cells were pretreated with the agent for 30 min before heat treatment (45 °C, 10 min). Data are expressed as means ± s.e.m. (*n* = 3–6): **P* < 0.05 versus unheated cells (Student's *t* test).

Treatment	(Concn.)	cAMP (fmol/10 ⁶ cells)	
		Unheated	Heated
Control		1858 ± 98	2475 ± 306*
Adenosine	(0.01 mM)	2500 ± 50	3750 ± 350*
	(0.05 mM)	3635 ± 15	6815 ± 445*
	(0.25 mM)	10525 ± 517	10923 ± 224*
AMP	(0.25 mM)	1725 ± 64	2375 ± 233*
ADP	(0.25 mM)	3683 ± 708	6250 ± 899*

Fig. 6. Heat does not cause additional increase in cAMP_i in cells treated with metabolic inhibitors

(a) Cells were exposed to 2-DG (10 mM), NaN₃ (10 mM) or DNP (1 mM) for 30 min, followed by heat treatment (■; 45 °C, 10 min) (*n* = 6). (b) Cells were treated with heat (■; 45 °C, 10 min), then treated with 2-DG (10 mM), NaN₃ (10 mM) or DNP (1 mM) (*n* = 6). cAMP_i was assayed in triplicate. Data are expressed as percentage of cAMP_i in control cells at 37 °C (■): **P* < 0.05 versus control at 37 °C; ***P* < 0.05 versus controls at 37 °C and 45 °C.

by the metabolic inhibitor. This is true, because the increase in cAMP_i stimulated by DNP (1 mM, 30 min) is diminished in the presence of IBMX (1 mM) from 4250 ± 200 to 3450 ± 50 fmol/10⁶ cells (both *n* = 3).

DISCUSSION

This study demonstrates that hyperthermia produces an increase in cAMP_i in human epidermoid carcinoma A-431 cells that is time-dependent. Heated cells recover their normal cAMP_i within 15 min after heating. Similar heat-induced increases in cAMP_i have been noted in human thymocytes [7] and rabbit epididymis [6]. Our experiments show that cAMP_i becomes lower than basal levels 1 h after heat treatment. This fall in cAMP_i is not related to cell death, because cells that are heated at 45 °C for 10 min remain viable (determined by Trypan Blue exclusion, ethidium bromide/Acridine Orange stain and lactate dehydrogenase measurement) and have the same plating efficiency as control cells 24 h after heat treatment [3]. However, previous reports have indicated that excess intracellular Ca²⁺ decreases the cAMP_i [27,31]. It is likely that this decrease in cAMP_i is secondary to the increase in intracellular Ca²⁺ induced by heat, because the latter increase is still observed 1 h after heat treatment (J. G. Kiang, unpublished work).

The heat-induced increase in cAMP_i is independent of external Ca²⁺ and Mg²⁺, which is similar to the result found in cells treated with epidermal growth factor [32]. In agreement with our

results, Ball *et al.* [32] reported that the basal cAMP_i is approx. 3000 fmol/10⁶ cells in either the absence or the presence of Ca²⁺ in A-431 cells.

Previous studies demonstrated that pH_i can affect cAMP_i [12–14]. However, in human A-431 cells, we found that an intracellular acidification produced by pre-pulses with NH₄Cl-containing Hanks solution does not change the baseline of cAMP_i. This observation is further supported by the results observed after amiloride treatment. On the other hand, it is known that cAMP_i can affect pH_i [8–11]. Application of 8-bromo-dibutyl cAMP (1 mM) to the cells for 10 min does not acidify the cells, whereas heating at 45 °C for 10 min induces a fall in pH_i of 0.2 pH unit [3]. It is known that intracellular acidification caused by heating is due to a combination of inhibitions of Na⁺/H⁺ exchange and metabolic pathways [3]. It should be noted that heat treatment at this temperature increases cAMP_i by only 850 ± 80 fmol/10⁶ cells. Because only extremely high concentrations of the cAMP analogue were capable of acidifying the cell, the much lower concentration of cAMP_i produced after heating is not related to heat-induced pH_i changes.

The increase in cAMP_i is prevented by the acidification induced by acid-loading or amiloride treatment, suggesting that pH_i modulates the activity of enzymes involved in cAMP formation [33,34]. Reynolds & Haugaard [35] reported that extractable phosphorylase activity in rat liver slices is significantly increased when external pH is 7.8. Another system that exhibits a relationship between cAMP_i and pH_i similar to A-431 cells is the noradrenaline-stimulated rat adipocyte, in which cAMP_i accumulation is dramatically depressed at external pH 6.6 compared with that at external pH 7.4 [36]. These data, together with data from acid-loading experiments, indicate that the thermal effect on cAMP_i occurs simultaneously with the intracellular acidification induced by heat [3].

To investigate the mechanisms underlying the heat-induced increase in cAMP_i, we examined the properties of phosphodiesterases, adenylate cyclase activity, G-proteins and ATP metabolites. Our experiments provide important clues about the mechanism through which heat stimulates an increase in cAMP_i. The observation that cAMP_i is increased after heating even in the presence of the phosphodiesterase inhibitors IBMX, Ro 20-1724 or theophylline excludes the possibility that heat inactivates phosphodiesterase. Three major observations support the view that the increase in cAMP_i is due to a combination of an activation of adenylate cyclase, G-proteins, and an increase in adenosine level. First, the direct measurement of adenylate cyclase activity in unheated cells and heated cells indicates that heat increases adenylate cyclase activity by a factor of 2.5 (Table 2). Second, heat attenuates the forskolin- or cholera-toxin-induced increase in cAMP_i, and the attenuation can be blocked by pretreatment with pertussis toxin, a G_i inhibitor (Table 1). This seems to indicate that heat promotes the interaction between adenylate cyclase and the G-proteins, particularly the stimulatory G-protein. The activity of G_i protein is detected when the activity of the stimulatory G-protein is maximized in the presence of either forskolin or cholera toxin. Third, the heat-induced increase in cAMP_i is attenuated by metabolic inhibitors that themselves can deplete the ATP pool and increase cAMP_i. Because cells pretreated with adenosine and exposed to heat do not show an additional increase in cAMP_i (Table 4), adenosine (presumably derived from ATP hydrolysis) probably stimulates adenylate cyclase activity in a manner that is inhibited by methylxanthines. This is further supported by data from the DNP experiments performed in the presence of methylxanthines.

Although cAMP is a positive regulator of epithelial-cell proliferation [37], the physiological function of elevated cAMP_i has not been studied in epithelial cells exposed to hyperthermia.

It has been shown that cAMP induces a dose-dependent reversal of the inhibition in protein synthesis induced by heat in murine leukaemia lymphoblast L5178Y, a non-epithelial cell line [38]. Actinomycin, which blocks the recovery of protein synthesis after heat shock, does not block the cAMP stimulation [39]. Boron *et al.* [8] demonstrated that cAMP applied internally to barnacle muscle fibres enhances the response of the pH_i-regulating system to acid loads. Therefore, it appears that an increase in cAMP_i induced by heat may be one of the reconstructive mechanisms that allows cells to recover from heat-induced dysfunction. However, some cell-culture studies indicate that an increase in cAMP_i stimulated by Ro 20-1724 or prostaglandin before, during or after heat treatment enhances the normal heat-responses of an inhibition of cell growth in murine neuroblastoma cells [40,41] and in murine B-16 melanoma cells [42]. It would be of interest to determine whether an application of 8-bromo-dibutyl cAMP directly to epithelial cells produces a synergistic effect on heat-induced inhibition of cell growth.

In summary, heat treatment increases cAMP_i in a time-dependent manner. The increase is dependent on pH_i, but is independent of extracellular Ca²⁺ or Mg²⁺. The increase in cAMP_i is due to a combination of activations of adenylate cyclase and G-proteins, and an increase in adenosine levels owing to ATP breakdown.

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