A novel pathway for adrenergic stimulation of cAMP-response-element-binding protein (CREB) phosphorylation: mediation via α_1 -adrenoceptors and protein kinase C activation

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Because of the central role of adrenergic mechanisms in the expression of crucial genes during brown adipocyte differentiation, we examined the activation (phosphorylation) of CREB (cAMP-response-element-binding protein) in mouse brown adipocytes in primary culture. We found that noradrenaline ('norepinephrine') stimulated CREB phosphorylation rapidly (maximum effect in ≤ 5 min with slow decay) and efficiently (EC_{50} , 6 nM). The increase in CREB phosphorylation coincided with increased expression of an artificial cAMP-response-element-containing reporter construct. CREB phosphorylation was partly inhibitable, both by the β -adrenergic antagonist propranolol and by the α_1 -adrenergic antagonist prazosin. Adenylate cyclase hyperactivation (by forskolin) could stimulate CREB phosphorylation to the same extent as noradrenaline. The α_1 -adrenergic agonist cirazoline also increased CREB phosphorylation. An

increase in intracellular $[Ca^{2+}]$ had, however, no effect, but protein kinase C activation by PMA was a potent stimulator. The cirazoline-stimulated (α_1 -adrenergic) CREB phosphorylation was inhibited by a desensitizing pretreatment with PMA, demonstrating that the α_1 -stimulation was mediated via protein kinase C activation; neither Src nor extracellular-signal-regulated kinases 1 and 2 activation was involved in the signalling process. We conclude that CREB phosphorylation in brown adipocytes is mediated not only through the classical β -adrenergic/cAMP pathway but also through a novel α_1 -adrenergic/protein kinase C/CREB pathway, which has not been described previously in any tissue.

Key words: brown adipocyte, cirazoline, noradrenaline, norepinephrine, PMA.

INTRODUCTION

The transcription factor cAMP-response-element-binding protein (CREB) is generally recognized as a key factor in the mediation of adrenergically induced gene expression [1,2], especially when the gene expression is induced via β -adrenergic receptors and mediated via an increase in cAMP and activation of protein kinase A. The effect of CREB activation (phosphorylation) should not be considered as being restricted to a few disparate genes. Rather, through its collective effects on a series of genes, activation of CREB may determine cell fate. Thus CREB activation is, for example, sufficient to induce differentiation (adipogenesis) in preadipocytes [3]. Concerning the differentiation process in brown adipocytes, CREB must be considered to be of particular interest, because a series of key genes are under positive adrenergic control (presumably at least partly mediated via CREB), one of them being the uncoupling protein-1 gene [4–9]. Further, cell proliferation and cell differentiation in general are positively regulated via adrenergic mechanisms in these cells (reviewed in [10,11]).

We have therefore examined adrenergically induced CREB phosphorylation in brown adipocytes. We found, as expected, that it was mediated partly via the β -adrenoceptor/cAMP pathway. However, we also observed an α_1 -mediated pathway for CREB phosphorylation (the first such observation in cells with endogenously expressed α_1 receptors), and established the existence of a novel pathway for CREB phosphorylation, through α_1 -receptors and protein kinase C activation.

MATERIALS AND METHODS

Animals, cell isolation and culture

Brown fat precursor cells were isolated from 4 week-old male mice of the NMRI strain, principally as described earlier [12], and cultured as described in [13]. The culture medium was Dulbecco's modification of Eagle's medium supplemented with 10 % newborn calf serum, 4 nM insulin, 25 μ g/ml sodium ascorbate, 10 mM Hepes, 4 mM glutamine, 50 i.u./ml penicillin and 50 μ g/ml streptomycin. One day after plating, the medium was changed to new fresh medium. The experiments were made routinely on day 4 after plating (if not otherwise stated), when the cells were pre-confluent. In some series, serum was omitted for the last 24 h prior to the experiments; this did not markedly alter the responses studied here, and the results have therefore been combined. All additions of drugs were made in volumes no larger than 1 % of the volume of the cell-culture medium.

Analysis of CREB phosphorylation by immunoblotting

After the indicated treatments, the medium was poured off and the cells were scraped into 60 μ l of hot SDS sample buffer [62.5 mM Tris/HCl, 2 % (w/v) SDS, 10 % glycerol, 50 mM dithiothreitol (DTT), 0.1 % (w/v) Bromophenol Blue and 1 $\times$ CompleteTM protease inhibitor cocktail (Boehringer Mannheim)] and thereafter sonicated. After boiling for 5 min, aliquots of 10 μ l were separated by SDS/PAGE (12 % gels) according to

Abbreviations used: CaM kinase, Ca^{2+} /calmodulin-dependent protein kinase; CRE, cAMP-response element; CREB, CRE-binding protein; Erk1/2, extracellular-signal-regulated kinases 1 and 2; MAPK, mitogen-activated protein kinase; pCREB, CREB phosphorylated at serine-133; DTT, dithiothreitol.

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Laemmli. Electrotransfer to a Hybond-C Extra membrane was performed with a semi-dry blotter apparatus. After transfer, the membranes were washed in Tris-buffered saline for 5 min, followed by quenching of non-specific binding (1 h at room temperature in 5 % non-fat dry milk/0.1 % Tween-20 in Tris-buffered saline). After quenching, the membranes were incubated with 1:1000 dilution of polyclonal phospho-specific CREB antibody (detecting CREB phosphorylated at serine-133; New England BioLabs) overnight at 4 °C. The primary antibody was detected with a secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit; New England BioLabs) and enhanced chemiluminescence (ECL; Amersham Biosciences). Detection was made by exposure of the membranes to HyperFilm™ ECL® (Amersham Life Science). The membranes were then stripped with 10 M urea, 50 mM sodium phosphate and 100 mM β -mercaptoethanol for 30 min at 50 °C and re-probed with a polyclonal control CREB antibody (New England BioLabs), detected with the same secondary antibody. The blots were again exposed to HyperFilm™ ECL™, and all films were quantified on a Molecular Dynamics densitometer. The ratios between phosphorylated CREB (pCREB; CREB phosphorylated at serine-133) and total CREB protein (pCREB/CREB) are shown in the results; to minimize the effects of background variation in the densitometer, the ratio was normalized in each experiment to that observed with 0.1 μ M noradrenaline ('norepinephrine').

Transient transfection and luciferase assay

Primary cell cultures were grown in 25 cm² flasks in the presence of serum until day 6, when they were transfected with the FuGENE reagent (Boehringer Mannheim) with 1 μ g of a commercial reporter construct, pCRE-Luc (PathDetect™; Strata-gene), consisting of four repeated cAMP-response element (CRE) sequences (AGCCTGACGTCAGAG; the CRE sequence is underlined) and a TATA box upstream of the luciferase gene. After 6 h, the medium was changed to fresh medium and the cultures were left overnight. The following day, where indicated, noradrenaline was added to the transfected cells, and after 6 h the cells were harvested in PBS. The cells were pelleted and lysed in 200 μ l of buffer containing 25 mM Tris/acetate, 2 mM DTT, 1 mM EDTA, 10 % glycerol and 1 % Triton X-100. For the analysis, aliquots of 100 μ l were diluted in 400 μ l of assay buffer containing 20 mM Tris/acetate, 33 mM DTT, 1 mM EDTA, 3.74 mM MgSO₄, 0.27 mM CoA, 0.47 mM luciferin (Sigma) and 0.53 mM ATP. Luminescence recordings were registered on a luminometer, principally by the method described in the manual for the Luciferase Assay System (Promega), and the results were expressed per mg of protein.

Analysis of cAMP levels

After stimulation of cell cultures for 5 min, the culture medium was aspirated and the cells were treated as described earlier [14,15]. Briefly, 0.8 ml of 75 % ethanol with 1 mM EDTA was added to each well and, after 10 min, the cells were harvested by scraping. Ethanol was removed by SpeedVac centrifugation and the pellets were suspended in 0.5 ml of 4 × TE buffer (40 mM Tris/HCl/4 mM EDTA in the final solution) and sonicated for 5 s. After centrifugation, a 25 μ l supernatant aliquot from each sample was used for determination of cAMP levels with the Cyclic AMP Assay System (Amersham Biosciences) according to the manufacturer's instructions.

Chemicals

The following chemicals were used to treat brown adipocytes: noradrenaline [(–)-arterenol bitartrate], D,L-propranolol, prazosin, isoprenaline, A23187, PMA and forskolin (all from Sigma), cirazoline, KN-93 and Ro31-8220 (RBI), PD98059 (New England BioLabs) and PP2 (Calbiochem). DMSO was used to dissolve A23187, PMA, forskolin, PD98059 and PP2; the final DMSO concentration per assay was maximally 0.1 %. This concentration had no discernable effect on basal or noradrenaline-induced CREB phosphorylation (104 ± 8 % of untreated). Ethanol (50 %) was used to dissolve prazosin; the final concentration per assay was maximally 0.5 %, which had no discernable effect on noradrenaline-induced CREB phosphorylation. Other chemicals were dissolved in water.

Statistics

The statistics were based on Student's *t* test, using mean values from duplicate wells in the indicated number of independent experiments. In the Figures, * indicate values significantly different from relevant controls, as detailed in the legends; # indicate values different from relevant induced level.

Figure 1 Noradrenaline-stimulated CREB phosphorylation

Brown adipocyte cultures, routinely on day 4 in culture, were stimulated with 0.1 μ M noradrenaline and analysed at different time points for CREB and pCREB content by SDS/PAGE and immunoblotting, as described in the Materials and methods section. (A) Representative image of immunodetected CREB and pCREB. (B) Time curve of the pCREB/CREB immunoreactivity ratio in noradrenaline-stimulated cultures after the indicated times. In each experiment, the ratio of pCREB/CREB at 10 min was set to 100%. The curve is based on values from two to five experiments performed in duplicate, given as means $\pm$ S.E.M. (C) Dose-response curve for noradrenaline (NE). The cultures were stimulated for 10 min with noradrenaline at the indicated concentrations and then analysed for CREB and pCREB content. In each experiment, the ratio of pCREB/CREB at 0.1 μ M noradrenaline was set to 100%. The points are based on values from three to six experiments in duplicate and given as means $\pm$ S.E.M. The curve is drawn for simple Michaelis-Menten kinetics (ignoring the 10 μ M point), yielding an EC₅₀ of 5.6 ± 1.2 nM noradrenaline. (D) Brown adipocyte cultures (day 6) were transfected with a reporter construct consisting of four CRE sites and the luciferase gene. The following day, the cultures were stimulated with 1 μ M noradrenaline for 6 h and the luciferase activity was measured. The results are based on values from three independent experiments, each performed in duplicate; in each experiment, the luciferase activity in the extract from the noradrenaline-stimulated cells was set to 100%. ****P* < 0.001 indicates a significant effect of noradrenaline compared with the control (C).

Figure 2 Effect of adrenergic antagonists on noradrenaline-stimulated CREB phosphorylation

Brown adipocyte cultures were stimulated for 10 min with 0.1 μ M noradrenaline (NE) alone or together with the receptor antagonists propranolol (Prop; 5 μ M) or prazosin (Praz; 5 μ M). In each experiment, the ratio of pCREB/CREB at 0.1 μ M noradrenaline was set to 100%. The graph is based on three experiments in duplicate and given as means $\pm$ S.E.M. *** P < 0.001 indicates a significant difference from the control (C); # P < 0.05 indicates a significant effect of antagonist treatment versus noradrenaline alone.

RESULTS

Adrenergically induced CREB phosphorylation

To examine the ability of adrenergic stimulation to activate CREB, we followed the phosphorylation of CREB in brown adipocyte primary cultures by immunodetection of total CREB protein and pCREB in cell lysates; the antibody used specifically recognized phosphoserine-133. As exemplified in Figure 1(A) (upper panel), CREB was present in the cells irrespective of whether they had been stimulated with noradrenaline or not. Almost no pCREB was detectable in unstimulated cells (Figure 1A, lower panel), but rapidly after noradrenaline stimulation a band appeared which remained visible for a prolonged time. To compensate for possible differences in protein loading, all blots were analysed with both anti-CREB and anti-pCREB antibodies as illustrated, and in the following, the activation of CREB is expressed as the ratio between pCREB and CREB, with the ratio 10 min after noradrenaline addition set to 100%.

Figure 1(B) shows the time course for CREB phosphorylation. As seen, CREB phosphorylation reached its highest value 5 min after the initiation of stimulation. The level then declined successively, but remained elevated for more than 1 h. In Figure 1(C), a dose-response curve for the effect of noradrenaline on CREB phosphorylation is shown. A rather high sensitivity to noradrenaline was evident, with an EC_{50} value of 6 nM.

In order to ascertain that adrenergic stimulation led to transcriptional activation via CRE elements, we transfected the brown adipocytes with a reporter system consisting of a plasmid with a short promoter, containing four CRE consensus sites, followed by luciferase. Although transfection efficiency in these primary cell lines was low compared with the efficiency in, for example, the brown-adipocyte-like transformed cell line HIB-1B (B. Leksell and B. Cannon, unpublished work), luciferase activity could be detected in the transfected cells. Further, as seen in Figure 1(D), 6 h of noradrenaline stimulation of the transfected brown adipocytes led to a significant increase in luciferase activity. (In other experiments, performed under similar condi-

Figure 3 Mediation of CREB phosphorylation

(A) Brown adipocyte cultures were stimulated for 10 min with 0.1 μ M noradrenaline (NE) or with 10 μ M forskolin (Fsk). In each experiment, the ratio of pCREB/CREB with 0.1 μ M noradrenaline was set to 100%. The graph is based on six experiments in duplicate and given as means $\pm$ S.E.M. (B) Brown adipocyte cultures were stimulated for 10 min with 0.1 μ M noradrenaline (NE), 1 μ M cirazoline (Cir), 1 μ M A23198 (A23) or 500 ng/ml PMA (TPA) [50 ng were also effective, but not 5 ng (results not shown)]. The graph is based on 6–9 experiments in duplicate and given as means $\pm$ S.E.M. The cirazoline-induced level was significantly lower than the noradrenalin-induced (P < 0.01). *** P < 0.001 compared with the control (C).

tions, we have observed that the increase in luciferase activity is not due to a general noradrenaline stimulation of protein synthesis; it is absent when other promoters are used, e.g. a modified C/EBP α promoter.) The increased transcription observed here is therefore probably mediated via the CRE sites.

Adrenergic receptors involved

To examine which type of adrenergic receptors mediated the noradrenaline-induced CREB phosphorylation, we initially employed adrenergic antagonists. CREB is classically considered to be the mediator of cAMP-mediated effects; in the adrenergic case here, the relevant receptors would be the β -adrenergic receptors. In agreement with this, the β -adrenergic antagonist propranolol, added in 50-fold excess over noradrenaline, led to an inhibition

Figure 4 Effect of the CaM kinase inhibitor KN-93

Brown adipocyte cultures were pretreated or not for 30 min with 10 μ M KN-93 and then stimulated for 10 min with 0.1 μ M noradrenaline (NE) or 1 μ M cirazoline (Cir). In each experiment, the ratio of pCREB/CREB for noradrenaline was set to 100%. The graph is based on values from three experiments in duplicate and data are given as means $\pm$ S.E.M. * P < 0.05 and ** P < 0.01 compared with the control (C).

Figure 5 Effect of protein kinase C inactivation

Brown adipocyte cultures (day 4) were treated or not with 500 ng/ml PMA (TPA). The following day, the cultures were stimulated for 10 min with 500 ng/ml PMA, 0.1 μ M noradrenaline (NE) or 1 μ M cirazoline (Cir). In each experiment, the ratio of pCREB/CREB with 0.1 μ M noradrenaline in cells that had not been pretreated with PMA was set to 100%. The graph is based on five experiments in duplicate and data are given as means $\pm$ S.E.M. * P < 0.05, ** P < 0.01 and *** P < 0.001 compared with the respective control (C). ns, Not significant compared with the control. Paired Student's t test yielded P < 0.01 comparing the PMA-pretreated NE effect with NE alone (#), and P < 0.05 comparing the PMA-pretreated cirazoline effect with cirazoline alone (#).

of the noradrenaline-stimulated CREB phosphorylation (Figure 2), but this inhibition was only partial. Notably, the α_1 -antagonist prazosin, added at the same concentration, led to a reduction of the same magnitude (Figure 2). This was a clear indication that α_1 -adrenergic receptors could also stimulate CREB phosphorylation in these cells (see also below). With the combined treatment of propranolol and prazosin, noradrenaline-induced CREB phosphorylation was practically abolished (results not shown; one experiment performed in duplicate). Further, the β -agonist

Table 1 Effect of α_1 -adrenoceptor stimulation on cAMP levels

Brown adipocyte cultures were stimulated for 5 min with 0.1 μ M noradrenaline, 1 μ M cirazoline or 500 ng/ml PMA, and cAMP levels were analysed as described in the Materials and methods section. Values are means $\pm$ S.E.M. from two experiments performed in triplicate. In each experiment, the noradrenaline value was set to 100% (corresponding to 1.4 ± 0.05 pmol/well). * P < 0.05, a significant effect of treatment compared with the control.

Treatment	cAMP (%)
Control	28 $\pm$ 12
Noradrenaline	100 $\pm$ 0*
Cirazoline	24 $\pm$ 6
PMA	30 $\pm$ 17

isoprenaline (1 μ M) stimulated CREB phosphorylation to a similar level as that observed with noradrenaline/prazosin treatment (not shown; four independent experiments), in agreement with the β -adrenergic stimulation of CREB phosphorylation earlier demonstrated in these cells [9,16,17]. Thus the observed CREB phosphorylation was composed of both the anticipated β -adrenergic component and an unexpected α_1 -adrenergic component. An α_1 -induced increase in CREB phosphorylation has not been reported before in systems with endogenously expressed α_1 -adrenoceptors.

Intracellular mediation

The β -adrenergic pathway is expected to be mediated via activation of adenylate cyclase and an increase in cAMP, leading to protein kinase A activation and probably direct phosphorylation of CREB [2]. To confirm that this pathway was functional, we examined the effect of the adenylate cyclase activator forskolin. Forskolin stimulation resulted in levels of phosphorylation similar to those observed after noradrenaline stimulation (Figure 3A), but higher than after selective β -adrenergic stimulation (Figure 2). This confirmed the functionality of the cAMP/CREB phosphorylation pathway in these cells.

To confirm that CREB phosphorylation could also be induced via α_1 -adrenoceptors, we first examined the ability of the selective α_1 -agonist cirazoline to stimulate CREB phosphorylation (Figure 3B; see also Figures 4 and 5). In agreement with the outcome of the antagonist (prazosin) experiments (Figure 2), cirazoline could induce a level of CREB phosphorylation that was about 50% of that induced by noradrenaline. To exclude the possibility that the cirazoline effect was due to unexpected increases in cAMP levels, we examined the ability of cirazoline to stimulate cAMP production in these cultures. As seen in Table 1, cirazoline had no effect on basal cAMP levels, whereas noradrenaline, as expected, had a marked stimulatory effect. Not even under optimal conditions for adrenergic stimulation of cAMP production in brown adipocytes (i.e. differentiated cells on day 6 in culture and about 20 min of stimulation [15]) did cirazoline have any effect on basal cAMP levels (results not shown; five independent experiments).

Intracellularly, α_1 -stimulation may be mediated in these cells via an increase in Ca^{2+} [13,18–20] and/or protein kinase C activation [21]. In examining the putative Ca^{2+} pathway, we found that the Ca^{2+} ionophore A23187 did not adequately mimic α_1 -stimulation, neither in itself (Figure 3B) nor in the augmentation of forskolin-stimulated CREB phosphorylation (results not shown). In other systems, Ca^{2+} -mediated CREB phosphorylation has been found to be mediated further via a Ca^{2+} /calmodulin-dependent protein kinase (CaM kinase), espec-

ially CaM kinase IV [2]. This kinase can be inhibited by KN-93 [22]. However, in accordance with the low potency of A23187 to mimic α_1 -stimulation, KN-93 was unable to inhibit α_1 -receptor-stimulated or noradrenaline-stimulated CREB phosphorylation (Figure 4). Thus the Ca^{2+} /CaM kinase pathway for CREB phosphorylation, recognized to occur in other systems [2], was apparently not mediating the α_1 -stimulated CREB phosphorylation in brown adipocytes.

In contrast, a very high level of CREB phosphorylation was induced by the protein kinase C activator PMA (Figures 3B and 5); PMA did not increase cAMP levels (Table 1). To examine whether protein kinase C was involved in the mediation of the α_1 -adrenergic signal, the brown adipocytes were pretreated with PMA for 16–20 h; this procedure is generally acknowledged to lead to down-regulation of (PMA-sensitive) protein kinase C activity [23,24]. In agreement with this, cells pretreated with PMA no longer responded to acute PMA treatment with an increase in CREB phosphorylation (Figure 5). Notably, PMA pretreatment had no effect on forskolin-induced CREB phosphorylation (results not shown). However, it halved the response to noradrenaline: the response was only $63 \pm 6\%$ of that in non-treated cells ($P < 0.01$). Pretreatment with the protein kinase C inhibitor Ro31-8220 had a similar effect (results from three independent experiments not shown). Notably, PMA pretreatment fully eliminated the response to the α_1 -agonist cirazoline (Figure 5). Thus the α_1 -stimulation of CREB phosphorylation was evidently mediated via protein kinase C activation.

Possible mediation via the Src/mitogen-activated protein kinase (MAPK) pathway

In vitro, both protein kinase A and protein kinase C can directly phosphorylate CREB on serine 133 [2]. However, the situation *in vivo* may be that downstream kinases are activated by these kinases, and it could be these downstream kinases that were responsible for physiological CREB phosphorylation [2,25,26]. This possibility could be of particular interest in brown adipocytes, where noradrenaline stimulation leads to MAPK activation [phosphorylation of extracellular-signal-regulated kinases 1 and 2 (Erk1/2)] [14,27–29] through both β - and α_1 -receptors; the two adrenergic pathways converge upstream of Erk1/2 at the level of activation of Src [29]. To examine the possible participation of the Src/Erk1/2 pathway in CREB phosphorylation, we used the inhibitor PP2 (10 μM) to inhibit Src, and the inhibitor PD98059 (50 μM) to inhibit Erk1/2 activation. Both inhibitors have been demonstrated earlier, under conditions similar to those used here, to be able to fully inhibit noradrenaline-induced Erk1/2 phosphorylation [14,29].

However, neither of these inhibitors affected the ability of noradrenaline to induce CREB phosphorylation in brown adipocytes; the noradrenaline-induced level was at least 85% of the level in cells without inhibitors (results not shown). Thus the β - and α_1 -adrenergic signals leading to CREB phosphorylation do not converge at the Src or Erk1/2 level, and these pathways are thus not involved in CREB phosphorylation. Rather, phosphorylation of CREB through β - and α_1 -adrenergic pathways may occur through direct phosphorylation by protein kinase A and protein kinase C, but, if not, the nature of any further kinase(s) involved in the mediation remains unclarified.

DISCUSSION

In the present investigation, we have demonstrated that in brown adipocytes CREB is phosphorylated as an effect of two parallel adrenergic pathways: one being the classical β -adrenergic/cAMP-mediated pathway, and the other being a novel α_1 -

Figure 6 Suggested model for the pathway for noradrenaline (‘norepinephrine’)-induced CREB phosphorylation in brown adipocytes

A sketch of the investigated intracellular signalling pathways for noradrenaline-induced CREB phosphorylation based on the experiments described here and earlier observations on Src and Erk1/2 activation [14,28,29]. See text for discussion. PKA, protein kinase A; PKC, protein kinase C; CaMK, CaM kinase.

adrenergic pathway, not mediated via Ca^{2+} and CaM kinases, but rather via activation of protein kinase C. This α_1 -pathway has not earlier been demonstrated in any cell type. The pathways examined and demonstrated are summarized in Figure 6.

The classical β -adrenergic/cAMP pathway

This pathway is involved in the noradrenaline-stimulated response, because the β -adrenergic antagonist propranolol could eliminate a fraction of the noradrenaline-stimulated CREB phosphorylation (Figure 2). That at least a fraction of the response is β -adrenergic is also the conclusion of studies on similar systems [9,16,17]. That the ensuing activation of adenylate cyclase is a sufficient step for CREB phosphorylation is clear, because forskolin could also stimulate CREB phosphorylation (Figure 3A). Concerning the mediation of the β -effects in these cells, it is clear that noradrenaline (and forskolin) leads to increases in cAMP levels [13–15,20,30,31] and thus to activation of protein kinase A [31], and protein kinase A probably phosphorylates CREB directly. Forskolin could induce a level of CREB phosphorylation that was higher than that of selective β -stimulation, indeed at least as high as that of total adrenergic stimulation (Figure 3A), and this may raise the question of why β -adrenergic stimulation could only generate a half-maximal response (Figure 2). However, forskolin can induce much higher levels of cAMP than those induced by noradrenaline [13,14], and forskolin can increase protein kinase A activity in these cells much more than can noradrenaline (H. Thonberg, J. M. Fredriksson, J. Nedergaard and B. Cannon, unpublished work, performed as in [31]). This supraphysiological effect of forskolin is thus probably the explanation for the ability of forskolin to increase

CREB phosphorylation more than was observed after selective β -adrenergic stimulation. The ability of forskolin to fully mimic adrenergic stimulation, misleadingly implying that all adrenergic stimulation of CREB phosphorylation is mediated via the β -adrenergic/cAMP pathway, may have masked contributions of non- β -adrenergic pathways in earlier investigations of adrenergically induced gene expression in brown adipocytes.

A novel α_1 -adrenoceptor/protein kinase C pathway

Notably, a significant fraction of the noradrenaline-stimulated CREB phosphorylation was mediated through an unexpected pathway: a novel α_1 -adrenoceptor/protein kinase C pathway. The ability of α_1 -receptors to stimulate CREB phosphorylation was evident from both antagonist and agonist studies (Figures 2 and 3B). This is the first report of α_1 -adrenergically stimulated CREB phosphorylation in a system in which the α_1 -receptor is expressed endogenously. The only previous report of α_1 -adrenergically stimulated CREB phosphorylation was observed in transfected systems, in which different α_1 -receptors were expressed ectopically in Rat1 or PC12 cells [32,33]. However, the α_1 -signal in the transfected Rat1 cells was demonstrated to be mediated through a cAMP/protein kinase A pathway [32]. Whereas the evidence for this pathway occurring in the transfected Rat1 cells is convincing [32], mediation of α_1 -signals through cAMP is not a pathway usually encountered in physiological systems. Indeed, all recent reports of α_1 -receptor-stimulated cAMP accumulation are the result of transfection studies with ectopically expressed α_1 -receptors, and the increase in cAMP is the indirect result of protein kinase C activation of adenylate cyclase (i.e. not directly through G_s protein) [34,35]. Earlier reports of a similar pathway in non-transfected cells report extremely low levels of α_1 -induced cAMP accumulation [36]. It has thus not been demonstrated that a α_1 -receptor/cAMP/protein kinase A/CREB phosphorylation pathway is functional in physiological systems. Particularly, in the cells used in the present study, α_1 -adrenergic stimulation, and protein kinase C activation by PMA, were clearly demonstrated not to lead to any increase in cAMP levels (Table 1; α_1 -stimulation may rather decrease otherwise elevated cAMP levels [13,20]). A cAMP pathway can therefore not be relevant for α_1 -signalling in brown adipocytes.

α_1 -Adrenergic stimulation of brown adipocytes leads to an increase in $[Ca^{2+}]_i$ [13,18–20]. In several other systems, increased $[Ca^{2+}]_i$ stimulates CREB phosphorylation, mainly through CaM kinase activation [2]. However, in the present study, an increase in $[Ca^{2+}]_i$ failed to stimulate CREB phosphorylation (Figure 3B). Therefore, the Ca^{2+} /CaM kinase pathway cannot be a major mediator of CREB phosphorylation. α_1 -Stimulation also leads to activation of protein kinase C [21], and artificial activation of protein kinase C by PMA is a very potent inducer of CREB phosphorylation (Figures 3B and 5). As such, an effect of protein kinase C activation on CREB phosphorylation has been observed earlier, particularly in lymphocytes [37–39]. However, that artificially induced protein kinase C activation can mimic adrenergic signalling (Figure 3B) is not in itself evidence that it is this pathway that mediates the α_1 -adrenergic response. Rather, it is the fact that pretreatment of brown adipocytes with PMA, leading to a complete inhibition of PMA-induced protein kinase C activity, also fully inhibited the α_1 -adrenergic response (Figure 5) which demonstrates that it is indeed via activation of protein kinase C that the α_1 -stimulated CREB phosphorylation is mediated in brown adipocytes.

Since protein kinase C, at least *in vitro*, can directly phosphorylate CREB [2], further mediation following protein kinase C activation may not be necessary. However, in several systems,

a mediatory role of MAPKs has been demonstrated, even to the extent that it is suggested that protein kinase A also does not act directly on CREB, but rather activates CREB through activation of mediatory MAPKs [2,25,26]. As the MAPKs Erk1/2 are activated in brown adipocytes via both β - and α_1 -adrenergic pathways [28,29], these kinases could be suggested to be a convergence point for β - and α_1 -adrenergic stimulation of CREB phosphorylation. However, verified inhibitors of Erk1/2 and Src activity in these cells [14,29] failed to decrease noradrenaline-stimulated CREB phosphorylation. This also probably excludes the involvement of MSK1 (mitogen- and stress-activated protein kinase 1) as an activating kinase for CREB [40], since MSK1 activation has been reported to proceed via MAPK [41]. It is therefore plausible that the coupling from activated protein kinase A and protein kinase C to CREB is direct in brown adipocytes, i.e. that these kinases phosphorylate CREB directly (Figure 6).

In conclusion, the present investigation, performed with brown adipocytes, provides evidence for a novel pathway in the activation of CREB, via adrenergic activation of α_1 -receptors and subsequent activation of protein kinase C. This pathway may, of course, also be relevant for the mediation of nuclear effects of α_1 -adrenergic stimulation in other cell types, the mechanism of which generally remains unknown [42].

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