most areas of adult brain[16,19], suggesting that they may contribute to cellular and synaptic differentiation in the developing central nervous system. Thus, the deficit of $\alpha 5$, $\beta 3$ and $\gamma 3$ subunits is likely to cause deleterious effects such as the tremor and jerky gait that appear in p^{cp} mice. Moreover, it is possible that a $\beta 3$ deficit leads to other development anomalies associated with p^{cp} (cleft palate and runting for example), because $\beta 3$ is expressed in germinal zones[16] and the mitotic zone of the forebrain[22]. Alternatively, the absence of other, as-yet unidentified, gene(s) in the p^{cp} deletion may be responsible for these phenotypes. Analysis of temporal and spatial expression patterns of $GABA_A$ receptor subunit, together with histological analysis of the developing p^{cp} brain, could provide an insight into the neurological and cellular basis of p^{cp} phenotypes, and also clarify the significance of $\alpha 5$, $\beta 3$ and $\gamma 3$ subunits in development.

The human counterpart of the region deleted in p^{cp} is associated with Angleman syndrome (AS)[5-9], which is characterized by severe mental retardation, microcephaly, seizures, ataxia, craniofacial anomalies and hypopigmentation[23,24]. The smallest known maternal deletion resulting in AS involves the $\beta 3$ but not the $\alpha 5$ gene[8,9] (although a single AS patient bearing a translocation is apparently intact for the $\beta 3$ gene[25]). Because the phenotypic effects of the p^{cp} mutation are recessive and independent of parental origin[2], the AS critical region may be outside of the p^{cp} deletion, despite certain phenotypic similarities between p^{cp} mice and AS patients[15]. However, if the mouse counterpart of the AS gene(s) is not imprinted, it is possible that the AS critical gene(s) may be within the p^{cp} deletion. Indeed, an AS-like paternal imprinting effect was not detected for the central region of mouse chromosome 7 (including the region deleted in the p^{cp} mutation[26]). Conservation of synteny of the region deleted in p^{cp} with human chromosome 15q predicts that the human $\gamma 3$ gene will map near the $\alpha 5$ and $\beta 3$ genes. It thus remains to be determined what role, if any, these three $GABA_A$ receptor subunit genes play in the aetiology of AS.

Note added in proof: Culiat *et al.*[32] have reported a concordance of $\beta 3$ gene disruption and cleft palate phenotype in a series of *p* allele deletions. □

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Amplification of calcium-induced gene transcription by nitric oxide in neuronal cells

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NITRIC oxide (NO) is a short-lived, highly reactive gas, which has been identified as a mediator in vasodilation, an active agent in macrophage cytotoxicity and neurotoxicity, and a neurotransmitter in the central and peripheral nervous systems[1-5]. Production of NO by neurons is critical for facilitated synaptic transmission in models of synaptic plasticity such as long-term potentiation and long-term depression, suggesting a role for NO as a retrograde messenger that could complete a hypothetical feedback loop by strengthening the connection between postsynaptic and presynaptic cells[6-10]. We report here that although alone NO has no evident effect on transcription, it can act as an amplifier of calcium signals in neuronal cells. NO and Ca^{2+} action have to coincide in time for amplification to occur. Experiments with a series of simplified reporter genes in combination with specific recombinant protein kinase inhibitors suggest that induction of gene activity following NO-amplified calcium action involves protein kinase A-dependent activation of the transcription factor CREB.

To model a situation in which a neuron receives a NO signal simultaneously with a signal of a different modality, we exposed neuronal PC12 cells to combinations of NO with different inducers of transcription and monitored changes in c-*fos* and c-*jun* messenger RNAs. These immediate-early genes serve as sensitive reporters[11], the modular structure of their promoter regions allowing correlation of incoming signals with discrete transcriptional regulatory elements and factors. Further, these genes encode components of a family of transcription factors that may mediate subsequent long-term changes in patterns of cellular gene expression[11]. Figure 1*a* shows that NO (produced by a nitric oxide-generating compound, sodium nitroprusside, SNP) was unable to induce transcription on its own; this holds true over a wide range (from 0.1 μM to 1 mM) of SNP concentrations (data not shown). But when NO was applied to cells in combination with certain treatments known to induce immediate-early gene expression, induction of c-*fos* expression was greatly enhanced in some cases. NO only amplified the action of those agents that act through calcium ions — ionophores A23187 and ionomycin, Bay K8644 (an agonist of voltage-gated calcium channels (VGCC), thapsigargin (an agonist of intracellular calcium release) and KCl, which depolarizes the cell membranes. In contrast, NO did not affect induction by forskolin or phorbol myristate acetate (PMA). NO had similar effects on c-*jun* induction, although to a much lesser degree. NO-mediated amplification was blocked by the Ca^{2+} chelator EGTA, by the VGCC

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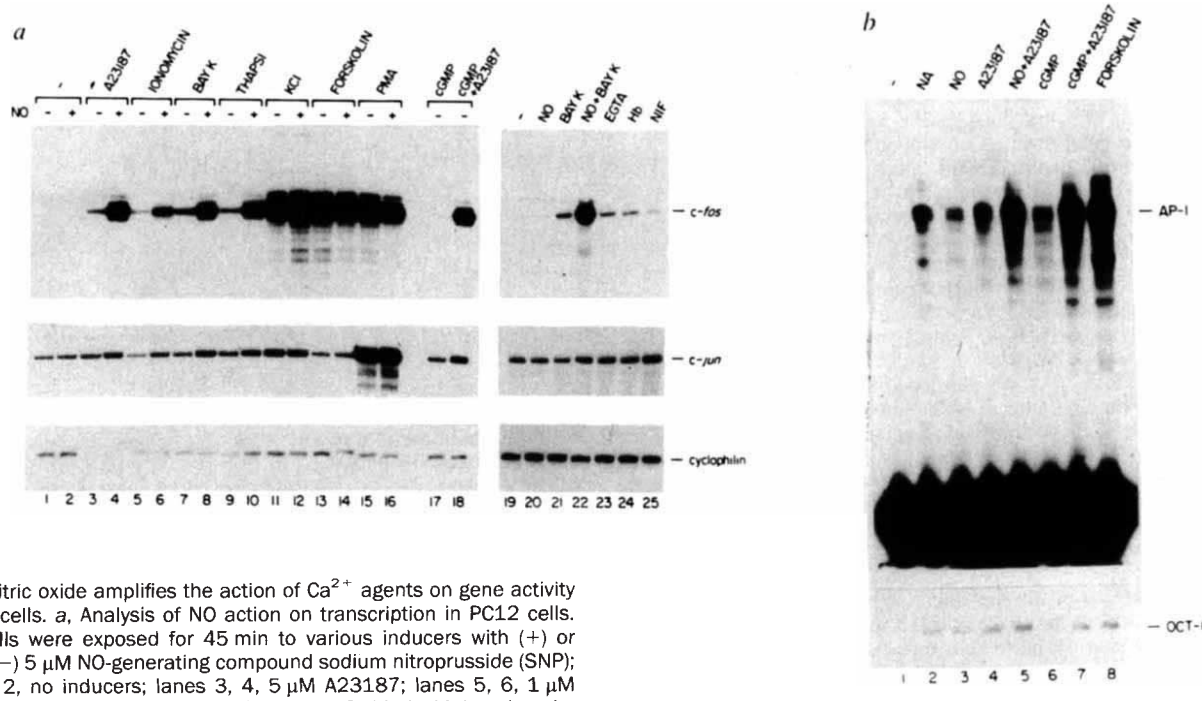

FIG. 1 Nitric oxide amplifies the action of Ca^{2+} agents on gene activity in PC12 cells. *a*, Analysis of NO action on transcription in PC12 cells. PC12 cells were exposed for 45 min to various inducers with (+) or without (−) 5 μM NO-generating compound sodium nitroprusside (SNP); lanes 1, 2, no inducers; lanes 3, 4, 5 μM A23187; lanes 5, 6, 1 μM ionomycin; lanes 7, 8, 5 μM Bay K8644; lanes 9, 10, 1 μM thapsigargin; lanes 11, 12, 55 mM KCl; lanes 13, 14 10 μM forskolin and 0.5 mM isobutylmethylxanthine (IBMX); lanes 15, 16, 200 ng ml^{-1} PMA; lane 17, 20 μM 8-Br-cGMP; lane 18, 20 μM 8-Br-cGMP and 5 μM A23187. Lanes 19–25 represent a separate set of experiments where the cells received no treatment (lane 19), 5 μM SNP (lane 20), 5 μM Bay K8644 (lane 21) or a combination of both (lanes 22–25), but were also treated with 3 mM EGTA (lane 23), 500 μg ml^{-1} of haemoglobin (lane 24) and 20 μM nifedipin (lane 25) 5 min before addition of SNP and Bay K8644. *b*, Enhancement of AP-1 binding activity by combined NO/Ca^{2+} action. Nuclear extracts were prepared from untreated cells (lane 2) and from cells treated with 5 μM SNP (lane 3), 5 μM A23187 (lane 4), a combination of both (lane 5), 20 μM 8-Br-cGMP without (lane 6) or with (lane 7) 5 μM A23187, or 10 μM forskolin and 0.5 mM IBMX (lane 8). Nuclear extracts were incubated with ^{32}P-labelled probes corresponding to the AP-1 binding site (AP-1) or the octamer motif (Oct-1) from the HSV ICP0 promoter.

METHODS. PC12 cells were grown in DMEM supplemented with 5% calf serum and 10% horse serum (HyClone). For induction, cells were exposed for 45 min to various inducers in the presence or absence of 5 μM SNP, dissolved in H_2O immediately before addition. Cells were collected on ice, washed with ice-cold PBS and the cytoplasmic RNA was extracted and analysed by RNase protection following standard procedures. The c-*fos* probe was prepared from a plasmid carrying mouse c-*fos* sequences from −56 to +109. The c-*jun* probe contained rat c-*jun* sequences from +295 to +527; cyclophilin probe (a gift from G. D'Arcangelo) contained rat cyclophilin sequences from +465 to +673. Rat c-*fos* mRNA carries three mismatches with the mouse probe, but under our T1 RNase digestion conditions it protected an RNA fragment of the expected length. The enhancement effect was reproduced by different sources of NO, including *S*-nitrosocysteine and 3-morpholino-sydnonimine (data not shown), suggesting that this effect is specific to NO. For band-shift analysis, PC12 cells were stimulated for 90 min, washed with ice-cold PBS and the nuclei were isolated by treatment with 0.1% NP-40. Nuclear proteins were extracted in high-salt buffer and binding assays were as described[14]. Synthetic oligonucleotides contained a single copy of AP-1 site (5′-TCGACGGTATCGATAAGCTATGACTCATCCGGGGGATC-3′) (a gift from K. Riabowol). Experiments with unlabelled wild-type and mutant AP-1 binding oligonucleotides and Fos- and Jun-specific antibodies confirmed that this elevation was due to specific binding by Fos- and Jun-containing protein complexes (data not shown). Treatment of PC12 cells with forskolin and IBMX produced the biggest increase in AP-1 binding activity and is reproduced (*b*, lane 8) for comparison with other inducers. For Oct-1 assays the same conditions were used except that each reaction contained 0.1 vol. of fetal bovine serum and the probe used was a 120 bp fragment containing a TAATGARAT element derived from the HSV ICP0 promoter (a gift from J. S. Lai).

antagonist nifedipin, and by extracellular haemoglobin, which binds NO and sequesters it from the media (lanes 23–25). The NO/Ca^{2+} induction of the endogenous c-*fos* gene and a transfected *fos* promoter showed rapid and transient kinetics characteristic of c-*fos* activation by a variety of inducers[11,12] (Fig. 2*a*).

The elevated induction of c-*fos* mRNA was productive, leading to an increase in binding activity of the AP-1 transcription factor comprising Fos and Jun family proteins. The combined action of NO and the Ca^{2+} ionophore A23187 produced a strong increase in AP-1 activity (Fig. 1*b*).

To test the possibility that NO acted by changing the level of cellular cyclic GMP[1–5], we treated the cells with a combination of A23187 and 8-Br-cGMP. Application of 8-Br-cGMP did not induce transcription, but, like NO, 8-Br-cGMP amplified the effect of Ca^{2+} influx (Fig. 1*a*, lanes 17, 18). This effect was also reproduced when AP-1 binding was measured (Fig. 1*b*). These data support the notion that cGMP may mediate NO action and indicate that cGMP can influence gene expression. Taken together, these results suggest that the combined action of NO and calcium leads to production of a highly active transcription factor capable of reprogramming the pattern of gene expression in neurons.

To determine whether NO and calcium act coordinately to produce a synergistic effect on transcription, we gave a pulse of one component, washed it away and added the second component immediately or after a further incubation of 5, 10 or 20 min. Addition of Bay K8644 after the removal of NO resulted in a loss of the synergistic effect (Fig. 2*b*, lanes 9–12). This was observed even when application of the two inducers was separated by only 5 min or less. After 10 min the signal was back to the levels of induction by Bay K8644 alone (compare lanes 3, 11 and 12). In the reciprocal experiment, in which Bay K8644 was applied first, a similar loss in the synergistic action of the two signals was observed (lanes 5–8). As a control, if the second reagent was added after 10 min without removing the first, the amplified response was nearly unaffected (lanes 13, 14). These

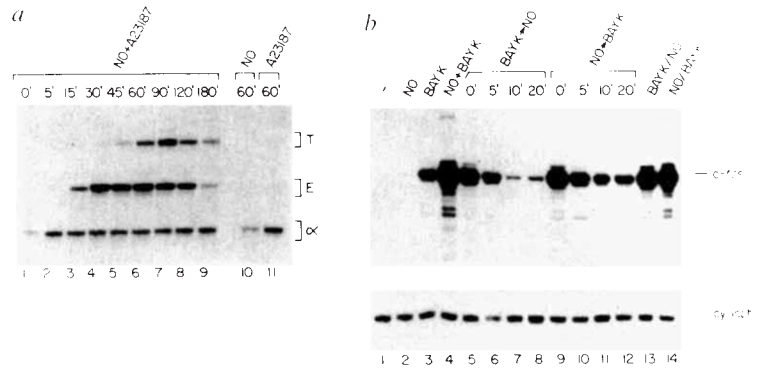

FIG. 2 Time requirements for the synergistic NO/Ca^{2+} action. *a*, Kinetics of induction of endogenous and transfected *fos* genes after NO/Ca^{2+} action. Reporter constructs containing *CAT* gene fused to the −356–+109 promoter region of c-*fos* gene (−356-*fos*-CAT) and a human α-globin gene used as an internal control were transfected into PC12 cells. After induction by 5 μM SNP and 5 μM A23187, RNA was isolated at various time points, indicated on the figure, and analysed by RNase protection assay as described for Fig. 1. T, E and α mark the positions of probe fragments protected by correctly initiated transcripts of the transfected c-*fos*-CAT plasmid, endogenous c-*fos* RNA and transcripts of the α-globin internal control, respectively. *b*, Analysis of the time window of the synergistic NO/Ca^{2+} action. PC12 cells were left untreated (lane 1), or were exposed to 5 μM SNP, 5 μM Bay K8644, or a mixture of SNP and Bay K8644 (lanes 2, 3 and 4, respectively). In a separate series of experiments (lanes 5–8) PC12 cells received a pulse of 5 μM SNP for 10 min, were washed several times with warm equilibrated DMEM, covered with warm equilibrated DMEM and, after indicated periods of time, received 5 μM Bay K8644 for 45 min; in reciprocal experiments (lanes 9–12) cells received a 10 min pulse of 5 μM Bay K8644, followed by washing, incubation and addition of 5 μM SNP after indicated periods of time. Lane 13, Bay K8644 (5 μM) was added to cells for 10 min followed by addition of 5 μM SNP for 45 min without removal of Bay K8644; lane 14, SNP (5 μM) was added to cells followed by addition of 5 μM Bay K8644 without removal of SNP. RNA was isolated and assayed for c-*fos* RNA as described for Fig. 1.

METHODS. Plasmids were transfected into PC12 cells by electroporation at 220 V, 960 μF. After a 40-h incubation the cells were stimulated for the indicated period of time, collected, and RNA was isolated as described in Fig. 1, except that RNA preparations were treated with RNase-free DNAase and purified by deproteinization. RNA was analysed by RNase protection assay using c-*fos* and α-globin-specific probes as described for Fig. 1. The α-globin probe contained human α-globin gene sequences from −15 to +95.

observations indicate that the two stimuli, NO and calcium, have to be present together within a small time window to exert their synergistic action.

Calcium induction of a transfected reporter gene composed of the c-*fos* promoter linked to the heterologous chloramphenicol acetyltransferase coding sequences (c-*fos*-CAT)[13,14] can also be amplified by NO, indicating that a short (400-base-pair (bp)) promoter region of the c-*fos* gene is sufficient to confer NO/Ca^{2+} inducibility (Fig. 2*a*). To examine which DNA sequences in the c-*fos* promoter and which transcription factors mediate this effect, we tested a series of simplified reporter constructs[13,14] (a gift from M. Gilman) for their ability to support NO-amplified calcium signalling. Promoter deletion analysis (Fig. 3*b*) shows two major transition points in induction levels, first when the reporter promoter was truncated from −356 to −275, which removed several important regulatory elements, and secondly when it was truncated from −71 to −56, removing the major cAMP-responsive element (CRE). These results are consistent

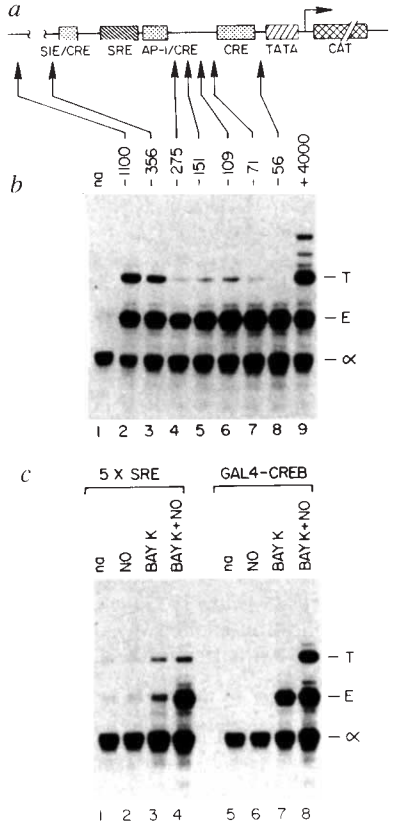

FIG. 3 CRE/CREB can transmit the amplified NO/Ca^{2+} signal to a reporter gene. *a*, Schematic diagram of c-*fos* upstream sequences. The regions corresponding to the *sis*-inducible element (SIE/CRE), the serum response element (SRE), the AP-1 binding site (AP-1/CRE), the cAMP response element (CRE), TATA box (TATA) and the CAT reporter gene are shown as filled boxes. *b*, Transient expression assay of mouse c-*fos* 5′-deletions. A series of reporter constructs containing truncated mouse c-*fos* promoter carrying sequences up to +109 and connected to the coding region of CAT gene[14] were transfected into PC12 cells along with the human α-globin internal control plasmid. Numbers and arrows above the lanes show the positions of 5′-end points of deletions of the c-*fos* promoter in c-*fos*-CAT fusions; lane 9 contained c-*fos* gene from position −356 to +4,000. *c*, Transient expression assay of multimerized SRE and of the GAL4/CREB reporter system. Cells were transfected with a reporter construct containing five copies of SRE connected to position −56 of c-*fos* promoter of the c-*fos*-CAT construct (5 × SRE, lanes 1–4), or with a mixture of 3 μg of reporter plasmid 5 × GAL4-CAT and 0.3 μg of expressor plasmid GAL4-CREB (GAL4-CREB, lanes 5–8) or its mutated (Ser 133→Ala) version (lanes 9–12). The constructs were the same as in ref. 20, except that GAL4-CREB fusion was expressed under the control of RSV promoter. All transfections contained α-globin internal control plasmid. Cells were transfected and RNA was assayed as described in Fig. 2. Series of truncated c-*fos*-promoter, 5 × SRE-CAT, 5 × GAL4-CAT and GAL4-CREB constructs were gifts from M. Gilman and L. Berkowitz.

with the function of the c-*fos* CRE as one, but not the sole, Ca^{2+} response element (refs 15, 16 and N.P., M. Gilman and G.E., manuscript in preparation). To test whether the c-*fos* serum response element (SRE), which can also transmit part of a Ca^{2+}-generated signal in KCl-depolarized PC12 cells (N.P. *et al.*, manuscript in preparation), is a target for the NO/Ca^{2+} pathway, we transfected cells with a reporter construct containing five copies of the SRE linked to a truncated c-*fos* promoter devoid of inducible sequences. Data in Fig. 3*c* (lanes 1–4) demonstrate that NO failed to amplify the calcium-dependent activation of this reporter. Thus, the SRE is not a target for NO-amplified Ca^{2+} signals.

CREs of several genes are bound by the transcription factor CREB[17–20]. To test directly the involvement of CREB as a downstream target of NO potentiation and to discriminate CREB-mediated effects from the action of endogenous transcriptional factors, we used effector proteins in which the DNA-binding specificity of CREB has been changed by fusion to the DNA-binding domain of the yeast transcriptional activator GAL4[20]. A reporter gene containing multiple GAL4 binding sites served as a sensitive indicator of the transcriptional activity of the hybrid CREB protein. The combined action of NO and Bay K8644 synergistically activated the reporter gene (Fig. 3*c*). A mutant form of GAL4-CREB lacking the Ser 133 phosphoacceptor site could no longer transmit the NO signal, thus demonstrating that a functional CREB protein is a target for at least one of the NO/Ca^{2+}-induced pathways in the nucleus.

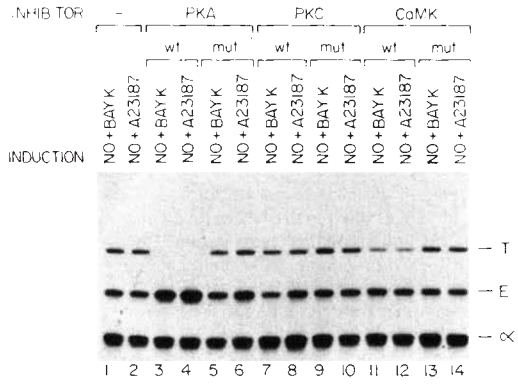

FIG. 4 Analysis of input of different protein kinases in NO-amplified calcium action. PC12 cells were cotransfected with −356-*fos*-CAT reporter construct along with 20 μg of recombinant inhibitors of different protein kinases and human α-globin control. Cells were exposed for 60 min to the action of 5 μM SNP and 5 μM A23187, or 5 μM SNP and 5 μM Bay K8644, as indicated, and RNA was isolated and assayed as described on Fig. 2. Reporter constructs were cotransfected either with pUC119 (lanes 1, 2) or with active (wt) and mutated inactive (mut) versions of recombinant protein kinase inhibitors as indicated. Lanes 3–6, recombinant inhibitors for protein kinase A (PKA); lanes 7–10, inhibitors for protein kinase C (PKC); lanes 11–14, inhibitors for Ca^{2+}/calmodulin-dependent multifunctional protein kinase II (CaMK). Recombinant inhibitors of protein kinases were expressed under the control of RSV promoter and rabbit β-globin splicing/polyadenylation sites. The structures of the recombinant PKC and CaMK inhibitors were matched to the sequences of the short conserved peptides located at positions 19–36 in various forms of PKC and positions 273–302 of CaMK, respectively. The recombinant PKA inhibitors included sequences of the murine PKA regulatory subunit RIα and the natural heat-stable PKA inhibitor (PKI). Such peptides act as powerful and highly specific pseudosubstrate inhibitors of the cognate enzymes *in vitro*[24–26]. Mutant forms of the inhibitors carrying inactivating amino-acid substitutions in the kinase recognition sequences served as controls. In a series of control experiments (N.P. *et al.*, manuscript in preparation and data not shown) the actions of recombinant inhibitors was shown to be selective for their target kinases and potent enough to block the reporter induction by a corresponding stimuli (forskolin, TPA, KCl).

Ca^{2+} signalling in neurons is mediated by several serine/threonine-specific protein kinases[21–23]. To determine which enzymes are involved in signalling by NO/Ca^{2+}, we have used specific recombinant inhibitors of individual protein kinases (N.P. *et al.*, manuscript in preparation). These inhibitors were constructed based on the autoinhibitory pseudosubstrate domains of several protein kinases[24–26] and they are potent and selective toward their cognate enzymes (N.P. *et al.*, manuscript in preparation). We cotransfected reporter plasmids carrying a full-length c-*fos* promoter with inhibitor plasmids and monitored whether selective inhibition of a particular protein kinase blocked NO/Ca^{2+} induction. Mutant inactive forms of the inhibitors served as controls. Consistent with the data on promoter mapping, only the cAMP-dependent protein kinase (PKA) inhibitor and, to a small degree, Ca^{2+}/calmodulin-dependent protein kinase II (CaMK) inhibitor blocked the NO/Ca^{2+} response (Fig. 4). These results implicate the PKA-CREB-CRE system as a major component of the signalling pathway for the transcriptional synergy of NO and Ca^{2+} and suggest that cAMP-Ca^{2+} synergism[15,27] may be a part of NO/Ca^{2+} signalling.

The phenomenon of NO-mediated potentiation of the Ca^{2+} response may have implications beyond transcriptional regulation. For example, NO could potentiate the PKA phosphorylation of cytoplasmic proteins with direct roles in synaptic function. The effect of NO on signalling might be particularly important at very low levels of calcium action, at which this inducer acting alone would have negligible effect; these very weak signals, which would go unnoticed by the cell, might be amplified by NO, resulting in pronounced physiological changes for the cell. Because NO diffuses freely, nearby synapses that receive very weak impulses simultaneously with exposure to NO might establish facilitated synaptic transmission[28]. NO and Ca^{2+} have to act within a very narrow time window for this enhancement to occur, suggesting that in the nervous system, this synergistic effect might be restricted to the recently active synapses, thereby coinciding with transient elevations of calcium levels[29]. □

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