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Drosophila NO-Dependent Guanylyl Cyclase Is Finely Regulated by Sequential Order of Coincidental Signaling

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Abstract We investigate the mechanism of regulation of *Drosophila*-soluble guanylate cyclase. Multiple putative sites of phosphorylation for the major kinases are present on both subunits of the heterodimer. We show that NO activation after binding to the heme group, is specifically modulated by sequential phosphorylations. PKA increases the NO stimulation at optimum level when both subunits are phosphorylated. Phosphorylation by CK (casein kinase-like) first, inhibits the PKA phosphorylation of the alpha subunit and limits the PKA upregulation of the cyclase activity. However, PKA phosphorylation first didn't prevent CK phosphorylation of the two subunits and the sequence PKA/CK induces higher level of NO activation than CK/PKA. These phosphorylations occur independently of NO binding and the direct inhibitory effect of calcium is observed for all the sCG forms. These data show that the sGC activity is regulated in a complex way, and the well-known asymmetry of the two subunits appears to cause the reading of the sequence of regulatory signals. This qualifies sGC as molecular detector on which converge coincidental and/or sequential neuronal signals. Furthermore, due to the fact that NO induction is huge (more than 600-fold obtained with the mammal counterpart), we might consider that any variation in kinases activation and/or calcium concentration in micro area of neuronal processes, provokes locally significant quantitative difference of cGMP synthesis in presence of diffusing NO. J. Cell. Biochem. 85: 392–402, 2002.

Key words: *Drosophila*; neurons; cyclase; cGMP; coincidental signaling

The natural behavior polymorphism due to a cGMP-dependent protein kinase of *Drosophila* has been well documented [Osborne et al., 1997]. Briefly, individual larvaes with the '*rover*' allele of the foraging gene move to a greater distance, meanwhile the '*sitter*' allele assignment confers strong sedentary posture in presence of food. Because the phenotype can be reversed from '*sitter*' to '*rover*' by overexpression of one isoform of the cGMP-dependent kinase, we aimed to investigate the effect of a specific component of the global cGMP: the cGMP produced by the NO-dependent guanylyl cyclase, on various integrative behavior of *Drosophila*. We reasoned that more associative is a specific behavior, i.e., is the result of multiple integrated neuro-sensorial signals, more sophisticated the molecular detectors of coincidental signals should be, as it has been already largely reported in literature on learning and memory [Bourne and Nicoll, 1993; Davis, 1996]. We were interested on sCG because, first, it is directly activated by NO through an heme binding [Kamisaki et al., 1986; Liu et al., 1995; Shah and Hyde, 1995], and second, this enzyme shows multiple potential sites of phosphorylation by different kinases. Moreover, authors have reported that the NO activation of the mammal counterpart is inhibited by calcium [Parkinson et al., 1999; Margulis and Sitaramayya, 2000]. Altogether, these elements are strong support for a "category" or "quality" of cGMP, which could be dissociated from cGMP generated by other types of cyclase.

Abbreviations used: PKA, protein kinase A, cAMP-dependent; PKG, protein kinase, cGMP-dependent; CK, caseine kinase-like; sGC, soluble guanylate cyclase; PKC, protein kinase C; NO; nitric oxide

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The subunits α1 and β1 of sGC present both putative sites for PKA and casein kinase II (CK II). The subunit α1 has, at least, three potential sites for PKA at the position 11, 317, and 437 and at least four potential sites for CK II 34, 50, 109, and 420. The subunit β1 presents, at least, PKA putative sites at position 32 and 529 and CK II putative sites at positions 283 and 368. We, therefore, determined whether the phosphorylation by these kinases, potentiates or inhibits the NO stimulation of the enzyme. We reasoned that there might be a synergistic, or at the opposite, an antagonistic effect of a particular kinase on NO effect, resulting in fine regulation of the cyclase activity. Cells permanently or transiently transfected with genes inserted vectors, was not appropriate to correlate the phosphorylation state and enzymatic activity, due to the fact that molecules nonfunctional like monomers, homodimers, heterodimers without the heme group are co-existing with the fully functional heterodimer coupled to the heme group, in a ratio difficult to address. We used immunoisolated material from fly extract to limit these inconveniences. We try, therefore, to determine how the phosphorylation pattern of sGC could change the enzymatic activity. Because *Drosophila* genetic is powerful to provide mutants null and/or hypomorph, which allows us to carry out behavioral testing, we analyze in this report the in vitro biochemical sGC activity in conditions that should mimic the resulting effect of coincidental and/or sequential order of signals in *Drosophila* neurons.

MATERIALS AND METHODS

Materials

PKA, PKC, and CK II were purchased from Sigma (P 7956, P 5511, and C3460, respectively). The antibodies against the alpha subunit sGC was a generous gift of Zuker's lab and Pak's lab, through a collaboration with Sokolowski's lab. The antibody against cGMP, anti rabbit agarose or Sepharose was also purchased from Sigma. *Drosophila* fly stock was maintained in standard conditions described elsewhere (*Drosophila*, a practical approach, D.B. Roberts, IRL Press, 1986) and flies were used between 5 and 7 days old. ^{32}P-GTP (3,000 Ci/mmol) and ATP (3,000 Ci/mmol) (alpha and gamma, respectively) were purchased from NEN life products. Hemoglobin (Sigma H 2500) was oxidized by air and sodium hydrosulfite followed by filtration on Sephadex column G-10 and used as NO scavenger. PMA (Phorbol 12-myristate 13-acetate) (P 8139) and H9 (*N*-(2-aminoethyl)-5-isoquinolinesulfonamide) (A 7795) were purchased from Sigma.

Strains Used in the Protocols

Dunce dnc^{1} [Nighorn et al., 1991] and *y dnc^{M14} cv^{1} v^{1} f1/FM7c* [Qui and Davis, 1993] and some 'smell blind' mutants: *olf A:X1*, *Sws olfx26*, *olf Cx3* [Carlson, 1991] come from the Bloomington *Drosophila* stock Center. *Hsp-Ala* and *Hsp-RQED* mutants are a generous gift from R. Greenspan's lab [Griffith et al., 1993; Broughton et al., 1996] and *rutabaga* mutant used was the classic strain described in literature [Levin et al., 1992]. *164* strain and *Hsp-Y2-2* are generous gift from M. Sokolowski's lab [Osborne et al., 1997]. Briefly, *dunce* shows strong increase of cAMP (eight-fold over the normal) and *rutabaga* is the opposite strain where the authors fail to observe elevation of cAMP after neurotransmitters release or calcium activation. *Hsp-RQED* overexpress after heat shock, a rat form of Cam kinase II, constitutively activated (the autophosphorylated serine is exchanged with an aspartate) and *Hsp-ALA* overexpress a pseudosubstrate inhibitory peptide for cam kinase II after heat shock. *Y2-2* overexpress an isoform of PKG (cGMP-dependent kinase dG2) after heat shock and the strain *164* is an hypomorph for this isoform. The strain *Hsp-sGC* overexpress the *Drosophila* dimer sGC after heat shock and is a generous gift from Marla Sokolowski.

Kinase Assay

The kinase assay was assayed by measuring the relative amount of P32 incorporated into specific substrate. The reactions were carried out with cold ATP (100 μM) or gamma ^{32}P-ATP (1 μM after isotopic dilution). In gel acrylamide analysis, we used a huge amount of kinases (see analysis of phosphorylated form of sGC) to overcome the limitation of the concentration of peptide consensus sequence of the target. For the PKC phosphorylation, diacylglycerol (DAG) and phosphatidylserine (PS) were added in the assay to activate the enzyme at the concentrations 0.8 and 8 μg/ml, respectively. DAG and PS were prepared by combining 50 μl of DAG in chloroform and 50 μl of PS and after chloroform evaporation, the mixture

was sonicated for 5 min in 500 μl of reaction buffer.

Preparation of the Fly Extract For Immunoprecipitation

Briefly, 100 flies were set on ice for 30 min, and the heads were cut off and ground in 20 mM Tris-HCl, pH 7.4, PMSF 1 μg/ml, aprotinin, and leupeptin 1 μg/ml. After centrifugation for 10 min at 10,000*g*, the supernatant was removed for the incubation with the antibodies at 4°C for 1 h. Then a second incubation with Sepharose beads attached to anti rabbit antibody or protein A was added for the time indicated in the following protocols.

Dosage of sGC Activity

One hundred flies were homogenized in ice, in 3 ml of 25 mM Tris-HCl, pH, 7.2, 1 mM EDTA, 2 mM EGTA, 1 mM dl-dithiothreitol, PMSF (1 μg/ml) leupeptin (1 μg/ml), aprotinin (1 μg/ml), and 0.005% Triton X-100 with a glass potter Duall. The extract was centrifuged at 4°C for 10 min at 10,000*g*. The supernatant was removed and incubated for 1 h at 4°C with three rabbit antibodies anti sCG (one from Liu et al., 1995, the other from Zuker's lab for the anti Dgc α, and the third raised from an expressed PCR product (sequence amino acids 252–550) in pGEX-1 to produce an hybrid protein with glutathione S-transferase, for the β subunit). The complex was then precipitated by an anti-rabbit agarose. The complex was dissociated in 50 μl of 200 mM NaCl, Tris 25 mM, pH, 7.2 at 30°C for 5 min, then diluted five times. The antibodies were simultaneously cleared from the supernatant by the fact that the anti-rabbit agarose shows still some affinity for the anti sGC at this salt concentration. An alternative was occasionally used and consists in attaching the antibodies directly to an activated agarose (*N*-hydroxysuccinimidyl-activated agarose, Sigma). On the other hand, no difference in phosphorylation and cyclase activity was observed when the sGC remains in presence of the antibodies and anti rabbit-agarose. Consequently, the experiments in this report, related to determination of activity, were done without elution. The dosages were then carried out at 30°C for the indicated time in buffer Tris 25 mM, pH, 7.2, 1 mM $MgCl_2$ and 1,000,000 cpm GTP (alpha ^{32}P) (3,000 Ci/mmol) and various concentrations of cold GTP and 1 mM of creatine phosphate and 1 U/tube of creatine phosphokinase with or without 0.1 mM sodium nitroprusside (for + NO or − NO). The dosage was performed as described elsewhere excepted that 1 mM of cold cGMP was added before the chromatography procedures. Briefly 500 μl of 120-mM zinc acetate was added to each tube then 500 μl of 144 mM of sodium carbonate. The mixture was precipitated by centrifugation, then the supernatant was further purified by acid alumina chromatography followed by sodium acetate (100 mM final) elution and the eluate was finally counted (see Domino et al., 1991). The activity is referred as milligram protein determined with total head extract. The cGMP measure was also performed with a variant of RIA procedure, specially the data presented in Figure 6. To stop the experiment, tubes are placed on ice and an antibody against cGMP (Sigma) was added and incubated for 30 min. Then the samples were complemented with serum albumin protein as carrier for the precipitation of the antibody (2% final). The samples then were precipitated by ethanol.

Analysis of Phosphorylated Form of sGC

The sGC was immunoisolated with two different polyclonal antibodies (from Zuker and Pak laboratories raised against an α subunit polypeptide and a 0.6-kb fragment of the cDNA, respectively) and a third antibody against a fragment 252–550 of the β subunit, and anti-rabbit agarose. The *Bolton Hunter* labeling of the isolated material shows a dimer with the two subunits of equal intensity (data not shown). The isolated material was directly phosphorylated by CK II (1 U/experiment according to Sigma), PKA (10 U/experiment according to Sigma), Cam kinase II and PKC (0.1 and 1 U/experiment, respectively according to Sigma) with gamma ^{32}P-ATP (2,000,000 cpm) (3,000 Ci/mmol) (isotopic dilution, 1 μM) as indicated for each experiment + cAMP (1 μM) for PKA, Ca^{++}/calmodulin (100 μM/1 μM) for Cam kinase II, phorbol ester (1 μg in 100 μl) for PKC, for the timing indicated for each experience. An alternative method was used, which consists in attaching the antibody to an activated agarose *N*-hydroxy-succinimidyl-agarose (Sigma), then the salt dissociated material (200 mM NaCl) was diluted (five-fold) and submitted to the same

protocols of phosphorylations. The phorbol ester treatment (5 μM) was carried out directly in the extract. The kinase phosphorylations were performed directly on the immunoprecipitate and/or alternatively on the immunoisolated material dissociated from the antibody as indicated above. In indicated circumstances, particularly when excess of cold ATP was eliminated prior to a second step of radioactive phosphorylation, the material was washed by centrifugal filter Amicon YM-10 twice and resuspended in cyclase activity buffer. The buffer for the cyclase and kinase assay was 20 mM phosphate, pH 7.5, EDTA 1 mM, PMSF 1 μg/ml, leupeptin 1 μg/ml, and bestatin 5 μg/ml. Leupeptin, bestatin, and EDTA was omitted in the buffer for kinase assay. The samples were finally incubated at 4°C for 1 hr with primary antibodies, then 20 min with of anti rabbit Sepharose or protein A-agarose. After brief centrifugation, the supernatant was discarded to eliminate the excess of radioactive ATP and the pellet was solubilized in loading buffer for gel acrylamide analysis. Alternatively, the samples were precipitated in trichloracetic acid (5% final), and the pellet was solubilized in loading buffer and neutralized with Tris base. No difference was observed between the two methods. In some experiments, short run of electrophoresis (20 min) (10% acrylamide) was carried out to measure the intensity of phosphorylation of the holoenzyme. The aim was to separate the sGC from the auto-phosphorylated regulatory subunit of PKA or other contaminants with low molecular weight. Gels were dried and submitted to autoradiography. The different acrylamide gels were dried and analyzed by autoradiography. The counts for enzymatic dosage were determined with a Beckman counter for beta radioelements. Fly Cam kinase II was affinity isolated using calmodulin-agarose and calcium. The kinase was released by EDTA and tested with a peptide substrate (Sigma C 4926) [Fong et al., 1989]. The PKG (cGMP-dependent kinase) from fly was isolated from Fly extract as described elsewhere [Osborne et al., 1997].

Quantification of sGC Protein

The extract of flies was dot blotted on nitrocellulose, then incubated with the antibodies. The relative measure of the protein was carried out with a second incubation with protein A labeled with *Bolton Hunter* (NEN), and the dots were analyzed by autoradiography or counted in a gamma counter.

RESULTS

NO-Dependent sGC Is Target for PKA and CK II

Figure 1 shows that the holoenzyme is substrate for PKA and CK II. Furthermore, Cam kinase (rat form or isolated fly form using affinity chromatography calmodulin-agarose) [Fong et al., 1989] shows any efficiency to add phosphate on sGC and the PKC efficiency seems low, although substantial. On the other hand, as expected the PKG (cGMP-dependent kinase) isolated from flies [see Osborne et al., 1997] failed to phosphorylate sGC (data not shown). The next step was to determine whether the NO binding to the heme group could facilitate or inhibit the phosphorylation process. NO seems to have any effect on the added phosphates on the holoenzyme by PKA and CK II as well (see Fig. 2). For both kinases, the dominant sub-

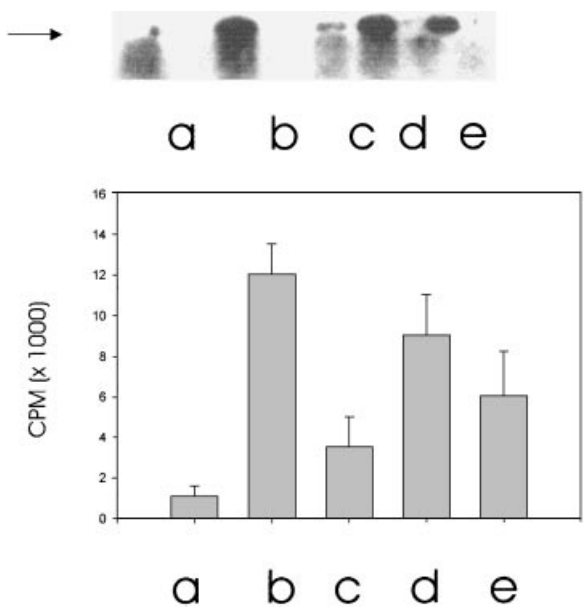


Fig. 1. Phosphorylation of the immunoisolated *Drosophila* sGC by different kinases. The immunoisolated material was phosphorylated by the indicated kinases as described in Materials and Methods with radioactive ATP. A full gel and a control without antibodies is shown in Figure 3. The material was then analyzed in 10% acrylamide gel for a short time (20 min) and the top bands quantified for their comparative intensity: (**a**) cam kinase II + Ca^{++} and calmodulin, (**b**) phorbol ester + cAMP + ^{32}P-ATP directly in the extract, (**c**) PKC + DAG/PS/Ca^{++}, (**d**) PKA + cAMP, (**e**) CK II. The quantification is shown below in the histogram (bars represent mean ± SE, n = 3).

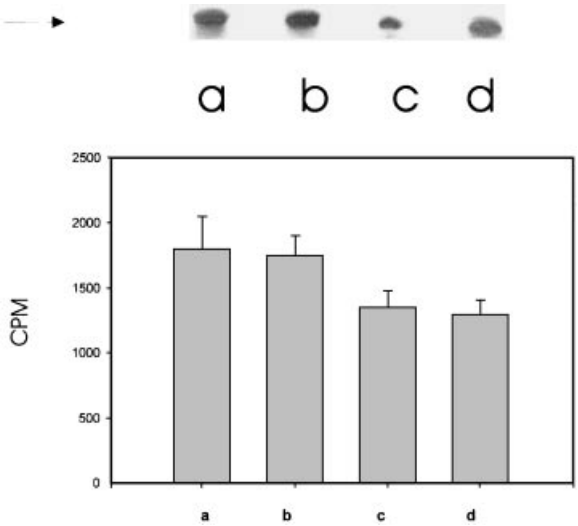


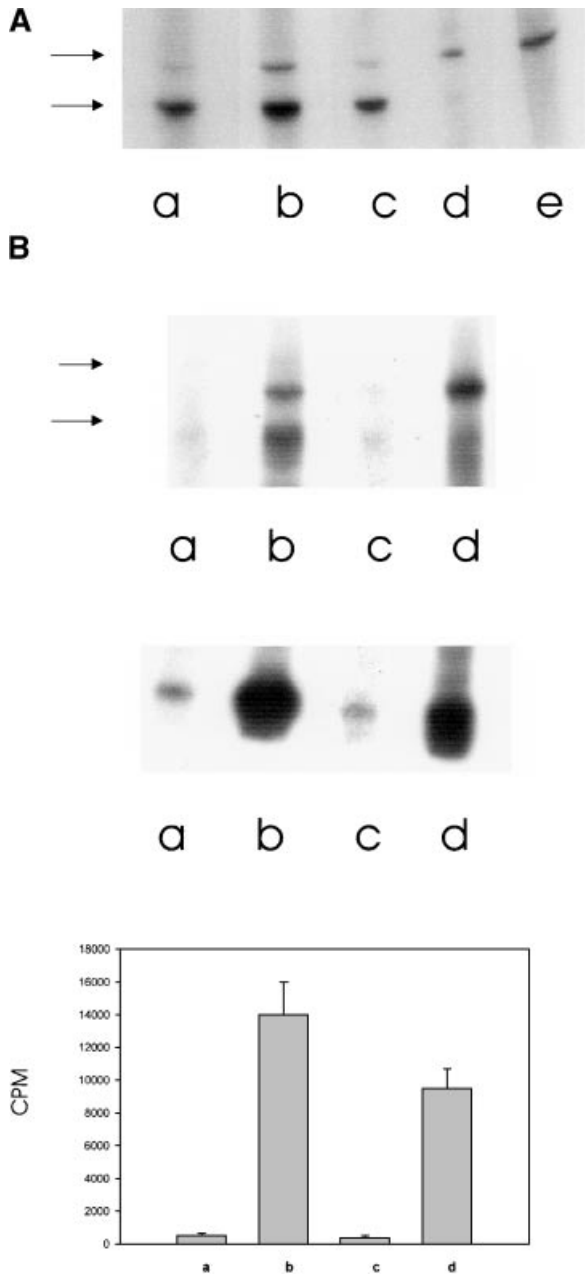
Fig. 2. NO effect on sGC phosphorylation by PKA and CK II. The immunoisolated material was phosphorylated with ^{32}P ATP in presence or absence of NO as described in Materials and Methods. The samples without NO were treated with oxyhemoglobin. A full gel and control without antibodies is shown in Figure 3. An acrylamide gel (10%) was run for a short run (20 min), dried, and analyzed by autoradiography: (**a**) PKA + sodium nitroprusside, (**b**) PKA, (**c**) CK + sodium nitroprusside, (**d**) CK. The quantification is shown below (bars are means ± SE, n = 3).

strate is the subunit α1 although longer exposition reveals intense β1 labeling. We observed that the previous phosphorylation of the holoenzyme by CK inhibits the PKA phosphorylation of the subunit α1, whereas the PKA phosphorylation of β1 seems unchanged (Fig. 3A). The presence of NO didn't change the profile of PKA or CK phosphorylation of the subunits when these phosphorylations were sequentially carried out after a first phosphorylation with cold ATP and CK II or PKA, respectively (Fig. 3B). Furthermore, we notice that PKA phosphorylation first didn't affect significantly the consecutive CK II phosphorylation at the difference of the reverse sequential order CK/PKA (Fig. 3B). We measured the activity of the immunoisolated sGC, sequentially phosphorylated by PKA/CK or CK/PKA. We used indirect measure, which consists in taking advantage of the cGMP produced to activate and added cGMP dependent protein kinase (PKG). We, therefore, analyze the induction of the PKG activity using the cGMP produced by the sGC immunoprecipitate submitted to phosphorylations as described above. An endogenous basic substrate about 12 kDa for PKG, trapped in the agarose beads, which presents the same physical characteristics than the bovine Histone H2a (determined by cation exchange chromatography using Carboxymethyl Sephadex and SDS gel electrophoresis) was used (see Fig. 3B, bottom). The PKG was isolated by affinity chromatography from fly head extract [see Osborne et al., 1997]. We observe that the PKA/CK phosphorylation sequence on sCG gives a relative higher level of kinase activity than the CK/PKA sequence. On the other hand, the prospect of fine upregulation effect of PKA on cyclase activity lead us to address the question whether the two subunits globally present the same relative affinity for PKA or alternatively whether this affinity is changing depending on previous phosphorylation by CK II. Using gradual concentration of PKA inhibitory peptide, we see clearly that the subunit α1 shows higher affinity than β1 for PKA (Fig. 4A). We noticed the presence of a higher band around 155 kDa, which might represent residual holoenzyme not completely dissociated. The lower band corresponds to molecular weight of the auto phosphorylated regulatory subunit of PKA. Furthermore, our data show that β1 presents roughly a range of affinity for PKA slightly weaker when the holoenzyme is previously phosphorylated by CK (Fig. 4B).

NO-Dependent sGC Activity Is Modulated by Coincidental and/or Sequential Phosphorylations

In Figure 5, we see that the PKA potentiates the NO effect, whereas CK II and Cam kinase shows no effect. Surprisingly, phorbol ester added directly in extract increases the NO stimulation of the enzyme, although the sGC holoenzyme seems to be a relatively poor substrate for PKC (see also Fig. 1). This suggests that PKC could act through associated proteins. Calcium, at the maximum concentration found in the vicinity of channels (100–200 μM), inhibits the sGC activity, as it has been reported with the mammal counterpart [Parkinson et al., 1999]. Furthermore, phorbol ester + br-cAMP added in the extract failed to increase synergistically or additionally the sGC activity (data not shown). The same result was obtained with immunoisolated sGC treated simultaneously with PKC and PKA (data not shown). Moreover, we consistently observed that the absence of NO donor gives a substantial residual volatile activity, which might be due to a substantial ratio of non-dissociated complex NO/heme in the extract. Authors have reported that the half-life of the complex NO/heme of the mammal enzyme, is about 90 min when the experiment is

carried out without scavengers [Brandish et al., 1998]. When NO scavengers were used (oxyhemoglobin), any residual activity was detected in the assay. Figure 6 summarizes the effect of sequential order of the two kinases PKA and CK on sGC activity. The data suggests, in accordance with the result in Figure 3B that CK II abolishes the stimulatory effect of PKA. Moreover, the reverse was not observed: PKA then CK II phosphorylation, in this sequential order, results in roughly the same enzymatic activity than PKA alone. We observed also that the use of NO scavenger (oxyhemoglobin) eliminates most of the activity after PKA and/or CK II phosphorylation (see Fig. 6, columns 4–6). Calcium, at the highest concentration in subcellular peak in neuron, inhibits partially the NO stimulation, whereas PKA treatment decreases the calcium inhibitory effect (see Fig. 6, columns 2, 3). However, these data were obtained with mammal (rat) CK II based on analysis of target consensus sequence, and therefore, we might consider that the *Drosophila* counterpart could give slightly different pattern of phosphorylation and/or PKA conjugated effect on NO-stimulated activity.



Dosage of NO-Dependent cGMP in Various Mutants of *Drosophila* Parallels the In Vitro Determinations

To verify the data related to the in vitro activity of the isolated forms of phosphorylated sGC, we performed a series of dosage of cGMP, NO-dependent with mutants presenting opposite effects in terms of neuronal cell signaling.

Fig. 3. **A**: In vitro phosphorylation of sGC by CK II and PKA. Effect of the coincidental and sequential order CK II/ PKA. The sGC was immunoisolated with three polyclonal antibodies and directly phosphorylated by CK II and PKA with ^{32}P-ATP for 10 min (**a**, **b**, **c**). The material was then again immunoisolated, then the material attached to anti rabbit Sepharose beads was solubilized in loading buffer and analyzed in electrophoresis 7.5% acrylamide gel (see procedure in Materials and Methods): (**a**) CK II, (**b**) PKA, (**c**) PKA + CK II. Furthermore, the immunoisolated material was phosphorylated by CK II, first, with cold ATP for 10 min, the excess of ATP was carefully washed out as indicated in Materials and Methods, then a second phosphorylation with ^{32}P ATP was carried out with PKA. This material was again immunoisolated to remove the excess of radioactive ATP and solubilized in the loading buffer: (**d**) CK II then PKA for 10 min, (**e**) CK II then PKA for 30 min. **B**: NO effect on the sequential phosphorylation of sGC: CK II/ PKA and PKA/CK II. High: The immunoisolated material was incubated with sodium nitroprusside (0.1 mM) and phosphorylated with either cold or ^{32}P-ATP as indicated in the procedure followed in Figure 3a. The arrows indicate the band at 75 and 85 kDa. A washing step was carried out to remove the excess of cold ATP before the second phosphorylation with isotopic ATP as above (see detailed procedure in Materials and Methods): (**a**) and (**c**) are controls without sGC antibodies, (**b**) PKA with cold ATP then CK for 30 min with ^{32}P-ATP, (**d**) CK with cold ATP then PKA for 30 min with ^{32}P-ATP. Bottom: In parallel experiment, the produced cGMP by sGC, phosphorylated in the same conditions as above was measured indirectly by the activation of added cGMP dependent kinase (PKG). Briefly, GTP (100 μM) and 10 μl of affinity isolated fly PKG were added with ^{32}P-ATP during the second phosphorylation step for 10 min. The labeled band corresponds to a fly endogenous PKG substrate of about 12 kDa: (**a**) and (**c**) are controls without sGC antibodies, (**b**) and (**d**) are phosphorylated sGC, respectively by the same sequence as described above. The quantification of the labeled PKG substrate is represented below (bars are mean ± SE, n = 3).

We used, therefore, *Dunce* (high level of cAMP) and *rutabaga* (low level), *RQED* (overexpressed Cam kinase II under heat shock promoter) and *ALA* (overexpressed inhibitory pseudo substrate of cam kinase II under heat shock promoter), and *Y2-2* (overexpressed PKG, the isoform dg2 of the cGMP dependent kinase, under heat shock promoter) and *164* (hypomorph for this kinase). The results are summarized in Table I, and show that high levels of cAMP increase the synthesis of NO-dependent cGMP. Our data confirm that Cam kinase has no effect on sGC activity, and the overexpressed PKG downregulates the production of NO-dependent cGMP by a mechanism, which is likely not a direct phosphorylation, but a more complex feed back as it has been suggested by some authors [Ferrero et al., 2000].

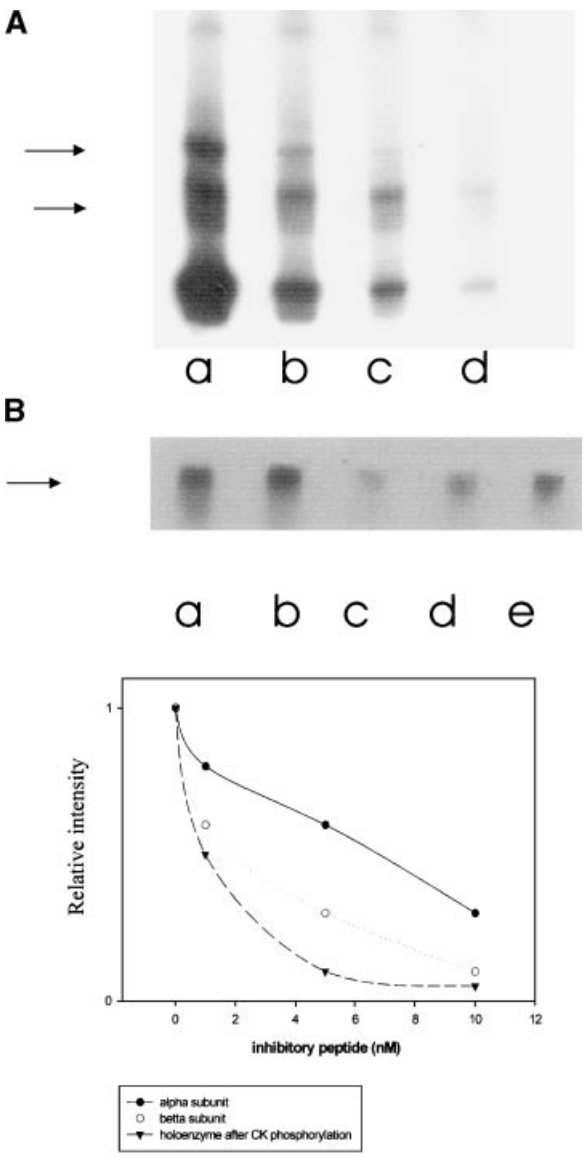


Fig. 4. **A**: Phosphorylation of sGC by PKA. Comparative affinity of the two subunits. We carried out an analysis of the relative affinity of the two subunits by displacement of the labeling by a PKA peptide inhibitor (P-0300 Sigma) (see procedure in Materials and Methods). ^{32}P-ATP and PKA were complemented with the different concentrations of peptide inhibitor. We see two major bands one at 75 kDa and the other at 85 kDa. A band at 155 kDa is also detected. The 85-kDa band shows lower affinity for PKA than the 75-kDa one. The lowest band (45 kDa) corresponds to the phosphorylated subunit of PKA: (**a**) control ^{32}P-ATP, (**b**) ^{32}P-ATP + 5 nM of peptide inhibitor (**c**) ^{32}P-ATP + 10 nM of peptide inhibitor, (**d**) ^{32}P-ATP + 100 nM of peptide inhibitor. The relative intensity of phosphorylated alpha and beta subunits obtained by density analysis of bands is shown below (each dot is the average, n = 3). **B**: Analysis of the relative affinity of sGC holoenzyme for PKA after CK II phosphorylation. The immunoisolated material was phosphorylated as indicated in Materials and Methods and analyzed on 7.5% acrylamide gel for a short run. The arrow represents species above 60 kDa, and therefore, should include the two subunits of sGC. The immunoisolated material was phosphorylated first by CK II with cold ATP, the excess washed, then a second phosphorylation was performed using PKA, ^{32}P-ATP and different concentrations of PKA peptide inhibitor, for 30 min (**c**, **d**, **e**). The material was then precipitated, briefly centrifuged, the radioactive supernatant discarded, and the pellet was analyzed in acrylamide gel for a short run: (**a**) control phosphorylation with CK II, (**b**) control with PKA, (**c**) sequential phosphorylation + 5 nM of inhibitor peptide, (**d**) sequential phosphorylation + 1 nM inhibitor, (**e**) sequential phosphorylation without inhibitor. The relative intensity is reported below (▲).

DISCUSSION

The N-terminal part of the Dgcα1 possess several potential sites for CK and one putative site for PKA in a linear sequence domain of about 100 amino acids. Another potential site for CK is close to another putative PKA site in the middle domain of the protein. Because of the proximity of these sites, we first speculate that the phosphorylation of one site by the relevant kinase will affect the efficiency of the other kinase on the next site. The data suggest strongly that the CK II added phosphates inhibit the phosphorylation by PKA on the Dgcα1. For the β subunit, the situation is more complex. First of all, a specific stretch of 180 amino acids, which doesn't exist in mammals, is present in the *Drosophila* form. This subunit is less phosphorylated by both PKA and CK II, compared to the Dgcα1, and the main point seems that when CK II phosphorylates the holoenzyme first, the PKA recognition site(s) on β subunit are not abolished although presenting

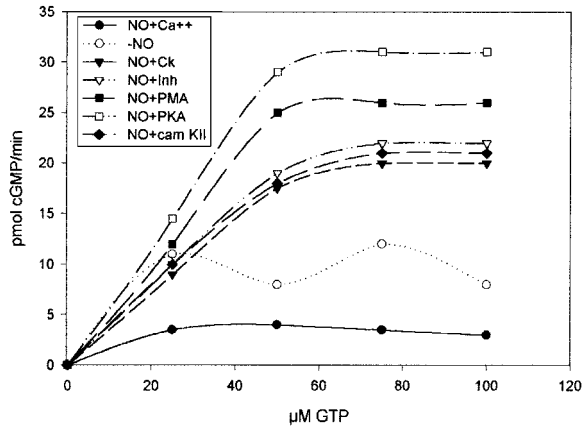


Fig. 5. Immunoisolated sGC activity. Effects of different kinases. The procedures of phosphorylation and cyclase activity determination are described in Materials and Methods. Calcium was used at 200 or 1 µM with calmodulin/Cam kinase. NO was produced by sodium nitroprusside at 0.1 mM. CK II and PKA are the rat CK II and bovine cAMP protein kinase. Inh is the PKA peptide inhibitor, used at 10 nM + H-9 used at 1 µg/100 µl. PMA, a phorbol ester was added at 1 µg/100 µl directly in the extract. Each plot is the mean of three different experiments (with triplicate for each determination). The activity is referred per milligram of protein of the extract prior immunoprecipation, and not to the amount of isolated sGC. No residual activity was detected when NO scavenger (oxyhemoglobin) was used in the assay.

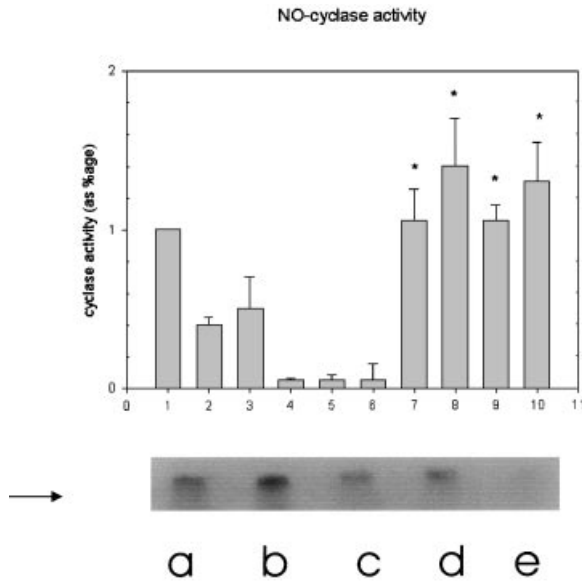


Fig. 6. Comparative effects of different phosphorylated states of sGC on NO-stimulated cyclase activity. sGC was immunoisolated and the phosphorylations were performed sequentially in the order indicated by the arrows. The cyclase activity was determined as indicated in Materials and Methods in presence of NO and expressed per milligram of total soluble head protein. Numbers are a percentage of the control and are expressed as the average of four determinations (mean ± SD). The numbers for the bars 7, 8, 9, 10 were determined from six separate experiments. Student's test, *: $P < 0.05$, **1**: + NO, **2**: + NO + 200 µM Ca^{++}, **3**: + NO + 200 µM Ca^{++} + PKA, **4**: + oxyhemoglobin, **5**: + oxyhemoglobin + PKA, **6**: + oxyhemoglobin + CK II, **7**: + NO + CK II, **8**: + NO + PKA, **9**: + NO, CK II→ PKA, **10**: + NO, PKA→CK II. Bottom: The first phosphorylation was performed with cold ATP, then the excess was washed out and the second phosphorylation was carried out with ^{32}P-ATP according to the protocols detailed in Materials and Methods (**c** and **d**). Bands represented in **a**, **b**, **c**, **d** correspond to the phosphorylation or sequential phosphorylation described respectively in the bar **7**, **8**, **9**, and **10**; (**e**) is a control without antibodies.

a lesser affinity. The consensus sequence for CK II presents acidic residues (glutamate and aspartate) in the vicinity of the phosphorylation site. PKA presents basic residue(s) in its specific target sequences. We might speculate that the negative charges of the acidic residues + the added phosphate group by CK II might interact by ionic bond with the positive charges of the basic residues of the PKA consensus sequence site, preventing its phosphorylation. Inversely, phosphorylation of the PKA sites, by adding negative charges should repulse the CK II sites, making them accessible for the enzyme. This hypothesis is corroborated by our experimental data. On the other hand, homologs of CK II in *Drosophila* have been identified, but little is known about their phosphorylation targets [Kloss et al., 1998; Suri et al., 2000]. The complex game between the two kinases seems, according to our data, to act differently on each subunit. This is interesting because monomers (α1 and β) are inactive, and therefore, the asymmetry of the heterodimer is a requirement for the catalytic activity [Shah and Hyde, 1995]. Moreover, authors noticed that the heme pocket is situated only in the N-terminal of the β subunit and the Dgcα1 don't possess heme moiety, although both subunits have a catalytic domain [Zhao et al., 1998]. Altogether, this suggests a sophisticated cooperativity between the two subunits, which is underlined by the catalogue of inhibitors and NO-independent activators acting on different sites of sGC [Ko et al., 1994; Garthwaite et al., 1995; Wu et al., 1995; Brunner et al., 1996; Schrammel et al., 1996; Becker, 2000; Severina et al., 2000; Zhao et al., 2000; Stasch, 2001]. Finally, although the β subunit possesses one putative site for PKG (cGMP-dependent kinase), we did not find solid evidence that this kinase phosphorylates the holoenzyme. This was expected, as auto amplification and/or inhibition of an enzyme by direct feedback triggered by enzymatic product or its

TABLE I. Comparative NO-Dependent cGMP Synthesis in *Drosophila* Mutants

Dnc M14 cvvf/FM7c	38 ± 7* pmol/ min/mg protein	1.25
Dnc 11	40 ± 8*	1.1
Rutabaga	19 ± 5*	0.98
Hsp-RQED8	28 ± 5	1.1
Hsp-ALA	25 ± 4	0.95
Hsp-RQED after heat shock	27 ± 6	—
Hsp-ALA after heat shock	27 ± 3	—
Hsp-PKG	21 ± 5	0.85
Hsp-PKG after heat shock	18 ± 7*	—
164	26 ± 4	1.2
Hsp-sGC after heat shock	43 ± 6	0.8
Cantonese	27 ± 6	1

Fly heads were grinded and the dosage of cGMP was carried out with the extract in presence of SNP (0.1 mM). The numbers correspond to the cGMP level after subtracting the basal level obtained without SNP. Three experiments were performed in triplicate (mean ± SD).
*$P < 0.05$ (Student's t-test) was determined vs. cantonese values. The column at the right represents the relative quantification of the sGC after nitrocellulose dot blot using antibodies incubation and protein A labeled with Bolton Hunter. The analysis was carried out by densitometry.

effector is very rare because such a mechanism is not appropriate to perform highly complex regulation.

Authors reported the stoichiometry one heme per heterodimer and huge elevation of the cyclase activity (up to 670-fold of the basal level) is triggered after NO binding [Brandish et al., 1998]. However, the co-transfection of the two genes coding for the two subunits resulting in overexpressed sGC in different type of cells, provide variable amount of increase from eight-fold to 200 hundred-fold after NO stimulation and successive purification steps are required to obtain fully active species. This shows that the limiting step in different expression systems tested is the fixation of the heme group in the pocket of β subunit, and therefore, the availability of the heme in the cytosol of the transfected cells [Yuen et al., 1994; Becker et al., 1999]. Very little is known about the heme binding, but the difficulties to fully reconstitute the sGC from an heme-deficient heterodimer suggests that the binding occurs during the folding of the protein [Margulis and Sitaramayya, 2000]. These facts argue for a direct immunoisolation of the protein in one step from *Drosophila* head extract in order to correlate the phosphorylated state of the subunits and levels of activity.

Furthermore, we detect significant amount of cGMP synthesized by sGC when NO donors were not used. This suggests that the endogenous NO has a strong affinity for the heme group when the cyclase is isolated. Authors have reported that the mammal counterpart presents a half-life of the complex NO/heme about 90 min [Brandish et al., 1998]. This explains also the variability of the assay without NO donors, and the fact that NO scavengers abolish the enzymatic activity. Our data suggest powerful *Drosophila* endogenous NO scavengers to prevent the enzyme of constant activation. This is also corroborated by the fact that the dosage in mutants, although in accordance with the in vitro determinations, provides significantly more difference in NO-dependent cGMP level. We might speculate that the phosphorylation state of sGC might influence the affinity for NO, which turns out to be difficult to analyze experimentally.

The phosphorylation state of mammal sGC has been already reported in literature where authors noticed that the level of activation correlate with the presence of phosphate group on the β subunit and the decline in NO stimulation is linked to phosphatase activity [Ferrero et al., 2000]. We think that there is cooperativity between the two subunits, which comes from their asymmetry, and therefore, they react independently to the complex game of kinases. The phosphorylation state seems independent of NO binding and the complex game of sequential order of activated kinases before the NO arrival, modulated the sGC activity after NO stimulation. Due to the fact that the NO induction is huge, the modulation effect is highly significant if we refer it to the basal level. After the diffusion of highly labile NO, local dendrites of the same neuron might show in parallel, substantial difference in sGC activation due to the local coincidental and/or sequential peaks of second messengers activating their specific kinases. This is strongly suggested by the finding that soluble guanylate cyclase is asymmetrically localized to the developing apical dendrite in mammals [Polleux et al., 2000]. This type of catalytic activity depending on the phosphorylation state is well known for some enzymes. This type of exclusive and conditioned phosphorylation has been already shown with dynamin, which is a GTPase enzyme, involved in the clathrin-dependent internalization of vesicles. The GTPase activity of dynamin is stimulated by PKC phosphorylation. CK phosphorylation abolishes the PKC recognition site, and consequently the activation of the enzyme [Robinson et al., 1993]. Interestingly, it is well known that calcium inhibits sGC [Parkinson

et al., 1999; Margulis and Sitaramayya, 2000]. Calcium and cGMP appear to have antagonistic functions in physiological systems like vascular smooth muscle [Moncada et al., 1991; Mülsch, 1997; Murad, 1999] and retinal photoreceptor cells [Koch and Stryer, 1988]. Our data show mainly that the modulation of sGC depends on coincidental signaling involving at least four major players: calcium, PKA, CK, and in some extend PKC + NO. This suggests that the activity can be deployed on a large spectrum of possibilities related to the order of arrival of these signals. The fact that NO induction is massive, any positive or negative modulation contributes to substantial differences compared to the level obtained with NO alone. Our data suggest that sGC is a molecular detector of coincidental signals, channeled by converging synapses on a micro-zone of a particular neuron.

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