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PHOSPHORYLATION OF PROTEINS IN CHICK CILIARY GANGLION UNDER CONDITIONS THAT INDUCE LONG-LASTING CHANGES IN SYNAPTIC TRANSMISSION: PHOSPHOPROTEIN TARGETS FOR NITRIC OXIDE ACTION

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Abstract—Production of nitric oxide and the activation of protein kinases are required for long-term potentiation of synaptic transmission at the giant synapses in chicken ciliary ganglion. In the present study, we investigated the ability of nitric oxide to regulate the phosphorylation of endogenous proteins under conditions that induced long-term potentiation in intact ciliary ganglion and the protein kinases responsible for the phosphorylation of these proteins in lysed ciliary ganglion. Using Calcium Green-1 we showed that the nitric oxide donor sodium nitroprusside did not change the intraterminal Ca^{2+} dynamics in ciliary ganglion. Two dimensional phosphopeptide analysis of $^{32}P_i$-labelled intact ciliary ganglion showed that the sodium nitroprusside (300 μM) increased the phosphorylation of several phosphopeptides (P50a, P50b and P41) derived from proteins at 50,000 and 41,000 mol. wts which we have called nitric oxide-responsive phosphoproteins. A similar stimulation of phosphorylation was achieved by 8-bromo-cyclic AMP (100 μM), which also induced long-term potentiation, but not by phorbol dibutyrate (2 μM) that does not induce long-term potentiation in ciliary ganglion. When subcellular fractions from lysed ciliary ganglion were labelled *in vitro* by [γ-^{32}P]ATP in the presence of purified cGMP-dependent, cAMP-dependent or Ca^{2+}-phospholipid-dependent protein kinases, we identified cyclic GMP-dependent protein kinase substrates that gave rise to phosphopeptides co-migrating with P50a, P50b and P41 from $^{32}P_i$-labelled intact ciliary ganglion. P50a and P41 were derived from soluble proteins while P50b was derived from a membrane-associated protein.

The proteins giving rise to P50a, P50b and P41 were also substrates for cyclic AMP-dependent protein kinase, but not for calcium and phospholipid-dependent protein kinase *in vitro*, suggesting that nitric oxide-responsive phosphoproteins are convergence points in information processing *in vivo* and their phosphorylation might represent an important mechanism in nitric oxide-mediated synaptic plasticity in ciliary ganglion. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: nitric oxide, protein phosphorylation, PKA, PKG, ciliary ganglion, long-term potentiation.

Long-term potentiation (LTP) is a long-lasting activity-dependent increase in the efficacy of synaptic transmission. Although Ca^{2+} is a common factor in different forms of LTP[3] the locations of its effects and

its targets differ. In the CA1 and dentate gyrus regions of the hippocampus the induction phase of LTP mainly depends on postsynaptic elevation of Ca^{2+} and activation of Ca^{2+}-dependent enzymes.[1,8,24,30,31,49,50] In contrast, at the mossy fibre terminals in the CA3 region of the hippocampus the induction of LTP occurs presynaptically and depends on the activation of adenylyl cyclase and cyclic AMP-dependent protein kinase (PKA).[20,38] The functionally important endogenous substrates for this protein kinase have yet to be identified.

Ciliary ganglion (CG) of the chicken contains only two types of neurons, the small choroid neurons and

the large ciliary neurons.[34] Ciliary neurons occupy about 60% of the total volume of the CG in day-old animals and receive a monosynaptic input via a giant nerve terminal, called a calyx, that contains multiple active zones.[18,33] This giant calyciform terminal is ideal for both imaging,[9,54] and electrophysiological studies.[52] In ciliary neurons high-frequency stimulation induces a form of LTP in which the efficacy of transmission is elevated for more than 30 min.[47] The induction of this LTP requires Ca^{2+} influx into the presynaptic terminal and results in an increased probability of release from the terminal, suggesting that the LTP is presynaptically induced.[48] Therefore, CG is an excellent system to investigate the mechanisms involved in presynaptic LTP.[28] As the long-lasting increase in synaptic transmission in CG can be inhibited by broad spectrum protein kinase inhibitors[47] our overall aim was to investigate the biochemical pathways leading to the induction of presynaptic LTP in this system.

Ca^{2+} and calmodulin (CaM)-activated systems have been shown to be essential in tetanus-induced LTP in CG.[47] It was hypothesized that Ca^{2+}/CaM-stimulated protein kinase II (CaMPK-II) may be one of the targets for Ca^{2+}/CaM action in CG. We have shown that CaMPK-II is present in CG[27] and is present in both the giant presynaptic nerve terminals and the ciliary neurons,[26] but the exact role of this enzyme has not been determined. Ca^{2+}/CaM can also activate nitric oxide synthase (NOS) and the production of nitric oxide (NO). Three lines of evidence directly implicate NO in the induction of LTP in CG: (i) NOS is present in the ganglion,[39] (ii) the NOS inhibitor L-NAME is able to inhibit tetanus-induced LTP,[47] (iii) LTP can be induced without tetanic stimulation by the bath application of the NO source sodium nitroprusside (SNP).[48] Both SNP- and tetanic stimulation-induced LTP share common intracellular pathways since potentiation by SNP inhibits the subsequent ability of tetanic stimulation to potentiate the synapses.[28]

The best understood mechanism for NO action is the activation of soluble guanylyl cyclase, resulting in the synthesis of cGMP.[19] The effects of cGMP can be to activate cyclic GMP-dependent protein kinase (PKG),[56] modulate cyclic nucleotide-gated ion channels[11] or modulate the activity of phosphodiesterases and therefore the level of cGMP and cAMP.[35] Direct application of the cell permeable 8-bromo-cyclic GMP (8-Br-cGMP) produces an increase in synaptic efficacy at these terminals without the need for tetanic stimulation[47] presumably via activation of PKG. Since CG has been shown to contain high levels of PKG activity[27] NO may act through the classical activation pathway stimulating guanylyl cyclase and then PKG, which in turn phosphorylates substrate proteins eventually leading to LTP. Using a combined approach of labelling intact[43,44] and lysed tissue[57] we set out to identify the proteins whose phosphorylation is changed in response to

NO and may be involved in the NO-induced LTP in CG.

EXPERIMENTAL PROCEDURES

Preparation of chicken ciliary ganglia

One- to five-day-old post-hatch white leghorn chickens (commercial meat breed, Australian Poultry, Beresfield, NSW) were used in all experiments. Ciliary ganglia (CG) were dissected as follows: after decapitation, the eyeballs were removed, the ganglia dissected and placed in 20 ml ice-cold Tyrode's buffer containing (in mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 3, glucose 10, HEPES 10 and $NaHCO_3$ 20. The buffer was gassed with carbogen (5% CO_2 and 95% O_2) for 40 min and then the pH was adjusted to 7.2–7.4 at room temperature using HCl. The Tyrode's buffer was then placed on ice and gassed with carbogen throughout the experiment.

Preparation of soluble and particulate fractions from ciliary ganglia

After decapitation CG were rapidly dissected (<1 min) and frozen in liquid nitrogen. Twenty-five frozen CG were hand-homogenized using a glass-glass homogenizer (Wheaton) in 200 µl ice-cold homogenization buffer (containing: 20 mM Tris–HCl (pH 7.7), 5 mM dithiothreitol, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 10 µg/ml leupeptin). Seventy-five microlitres of the homogenate was removed and diluted twofold with an equal volume of homogenization buffer ("total" fraction). The remaining homogenate (125 µl) was then centrifuged at 30,000 g at 4°C for 25 min (Beckman Optima TLX ultracentrifuge). The supernatant was collected as the "soluble" fraction. The pellet was resuspended in 125 µl of the homogenization buffer, rehomogenized, and called the "particulate" fraction.

Protein phosphorylation of intact ganglia

Phosphorylation of intact ganglia was performed as described,[43,44] with the modification that 10 CG were pre-incubated in 240 µl of Tyrode buffer for 60 min in the presence of 1 mCi/ml $^{32}P_i$ at 37°C with continuous oxygenation with humidified carbogen. Then 10 µl of 50 µM phorbol dibutyrate (PDBu), 2.5 mM 8-Br-cAMP or 7.5 mM SNP (resulting in a final concentration of 2 µM, 100 µM or 300 µM, respectively) in the presence or absence of 10 µM Ro31-8220 were added for a further 10 min (PDBu and Ro31-8220 were dissolved in dimethylsulphoxide (DMSO) and diluted with Tyrode's buffer to a final 0.1% DMSO content, and sonicated; 8-Br-cAMP and SNP were dissolved in Tyrode's buffer). The reactions were terminated by the removal of incubation solutions and the addition of 90 µl sodium dodecyl sulphate (SDS) sample buffer and immediate boiling for 5 min to solubilize the proteins. Aliquots (80 µl) of solubilized proteins were analysed by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) which were then stained, destained then dried between cellophane sheets and exposed to X-ray film. These conditions were similar to the conditions used in the electrophysiological experiments of Scott and Bennett[47,48] to produce a maximal increase in excitatory postsynaptic potentials (EPSPs), except that the temperature was 37°C in our experiments.

Protein phosphorylation in subcellular fractions

Phosphorylation reactions were performed by incubating subcellular fractions at 30°C with [γ-^{32}P]ATP at 0.2 µCi/µl for 1 min. The incubation mixture (in a final volume of

100 µl) contained 30 mM Tris–HCl (pH 7.4), 1 mM EGTA, 10 mM MgSO$_4$, 40 µM ATP, and 0.05% Tween 80 in the absence or presence of 1.2 mM CaCl$_2$, 40 µg/ml of phosphatidylserine (PS), 4 µg/ml of diacylglycerol (DAG), 10 µM cGMP or 10 µM cAMP as indicated. PKA inhibitor peptide PKI (final concentration 0.01 mg/ml) was routinely added to the phosphorylation reactions for basal phosphorylation and the conditions used to stimulate PKG or PKC to prevent cross-activation of any endogenous PKA. After 5 min pre-warming to 30°C, the phosphorylation reactions were initiated by addition of 20 µl (100 µg) ice-cold CG fraction followed immediately by the purified protein kinase (in 10 µl). The protein kinases were all used at 40 ng per tube and were purified according to the procedure of Robinson et al.,[45] diluted immediately before use in 5 mM Tris–HCl (pH 7.4) containing 0.05% Tween 80, and were kept on ice (up to 15 min) and discarded at the completion of the experiment. Phosphorylation reactions were terminated by the addition of 50 µl SDS sample buffer. Proteins were separated on 1-mm-thick 20-cm-long 10% polyacrylamide gels (Protean II system; Bio-Rad) and the phosphoproteins were visualized by autoradiography.

Phosphopeptide analysis

Following the separation of ^{32}P$_i$-labelled intact CG or [γ-^{32}P]ATP labelled subcellular fractions on 10% SDS–PAGE, the region of interest was cut from the gels (Fig. 1, dashed line). The gel strips were then rehydrated in a buffer containing 125 mM Tris–HCl (pH 7.4), 0.1% SDS and 2% mercaptoethanol and the cellophane was removed. The gel strips were loaded onto a 20% acrylamide gel for further separation. For the whole track digestion 250 µl of 50 µg/ml *Staphylococcus aureus* V8 protease (where V8 was dissolved in 125 mM Tris–HCl (pH 7.4), 0.1% SDS, 2% mercaptoethanol, 30% glycerol) was loaded onto the wells. Proteins were run half way into the stacking gel together with the V8 protease, the power was switched off for 45 min to allow phosphopeptides to be generated by partial digestion of the phosphoproteins. The phosphopeptides were separated by electrophoresis at constant current. The gels were dried after a 10-min fixation in 7% acetic acid and the phosphopeptides were visualized by autoradiography. The autoradiograms were analysed by a computer-aided densitometer (Molecular Dynamics). Phosphorylated peptides were designated according to the apparent molecular mass of the protein from which they were derived.

Ca^{2+} imaging in the calyx

Calyciform terminals were specifically loaded by orthograde transport of the calcium indicator, Calcium Green-1 (10,000 mol. wt) as previously described.[4] Laser scanning confocal microscopy allowed the recording of the [Ca^{2+}] response within a single terminal to single impulse stimuli with a temporal resolution of 220 ms. The bath solution contained 5 mM Ca^{2+} in order to increase the amplitude of response for single impulse stimuli. Calcium concentrations were estimated as described by Brain and Bennett.[5] Briefly, the only parameter that needs to be measured in order to correct for the non-linearity of the dye is the ratio of the maximum fluorescent intensity to that at the resting calcium concentration. This parameter was measured by providing a very long train of high-frequency stimuli (30 Hz), allowing the high [Ca^{2+}] to saturate the dye. It was found to be 4.8 ± 0.5 (n=4). In order to estimate the absolute value of the Δ[Ca^{2+}] it is necessary to estimate K_d and the ratio of the maximum to minimum fluorescent intensities; these were, respectively, 243 nM[10] and 14 (Molecular Probes Handbook, available from http://www.probes.com/handbook/toc.html).

RESULTS

Sodium nitroprusside does not stimulate Ca^{2+} entry into the calyciform terminals

Our previous experiments showed that tetanic stimulation induced a form of LTP in CG by a pathway that requires Ca^{2+} entry, calmodulin and protein phosphorylation and that the pathway involved in SNP-induced LTP shared at least some steps with that involved in tetanus-induced LTP.[28,47] We sought to identify at which point along this pathway SNP was having its effect. One possibility was that SNP might increase the intracellular Ca^{2+} concentration in the calyx. Although previous observations showed that SNP decreases the intracellular Ca^{2+} concentration in cultured dissociated ciliary neurons,[7,23] embryonic CG[4] and rat brain synaptosomes,[36] it was possible that SNP may alter the intracellular Ca^{2+} concentrations achieved following the arrival of an action potential.

In order to investigate the effect of SNP on Ca^{2+} influx, calyciform terminals of one- to two-day-old chicks were orthogradely loaded with the Ca^{2+} indicator Calcium Green-1. The addition of increasing concentrations of SNP (to up to 1 mM) to the bathing solution caused no measurable change in the resting Ca^{2+} concentrations, as indicated by a steady resting fluorescent intensity from Calcium Green-1 (data not shown). After 15–20 min in 1 mM SNP single impulse stimuli were applied to the presynaptic nerve. There was no increase in the amplitude, the rate of decay or the duration of the change in Ca^{2+} concentration in the calyx following single impulse stimuli compared to the control values in the same terminal (Table 1; n=4). The SNP was then removed by perfusing the ganglia with normal bath solution for 20 min. The subsequent application for 5–10 min of a solution containing 10 mM Ca^{2+} caused a significant increase in the amplitude (40 ± 13%; two-tailed Student's t-test, $P<0.05$, n=4), but not the rate of decay or the duration of the increase in intracellular Ca^{2+} concentration in response to single impulse stimuli (Table 1). This suggests that, had the SNP caused a significant increase in Ca^{2+} influx, the indicator dye should have detected it. These results indicate that SNP induces LTP by acting at a step not involving Ca^{2+} entry into the terminal.

Protein phosphorylation in intact ciliary ganglion

Previous experiments have shown that bath application of SNP, 8-Br-cAMP or 8-Br-cGMP to isolated intact ganglia induced LTP without tetanic stimulation, whereas bath application of PDBu altered post-tetanic potentiation, but did not induce LTP.[47] It has also been shown that activation of protein kinases is involved in the induction of LTP in CG.[47] Taken together, these results suggested that PKG and/or PKA, but not PKC activation is involved in LTP induction. To identify the protein kinases, and their

Table 1. The effect of sodium nitroprusside on intracellular concentration of Ca^{2+} in the presynaptic calyx following stimulation by a single impulse

	Amplitude (nM)	Time constant of decay (ms)	Duration (ms)
Control	28.54 ± 1.11	182 ± 21	545 ± 63
1 mM SNP	27.59 ± 1.01	219 ± 28	656 ± 84
High Ca^{2+}	$45.41 \pm 2.97^*$	183 ± 14	548 ± 42

The change in Ca^{2+} concentration within a single calyciform terminal was measured following a single impulse stimulus to the preganglionic nerve. Each value is the mean $\pm$ S.E.M. of recordings from four consecutive stimuli, each 2 min apart in quadruplicate. The duration of Ca^{2+} influx was given as the time required for maximal amplitudes to decay to 95% of their pre-stimulus values. The fluorescence response in each case was corrected by subtracting the average of three control recordings.

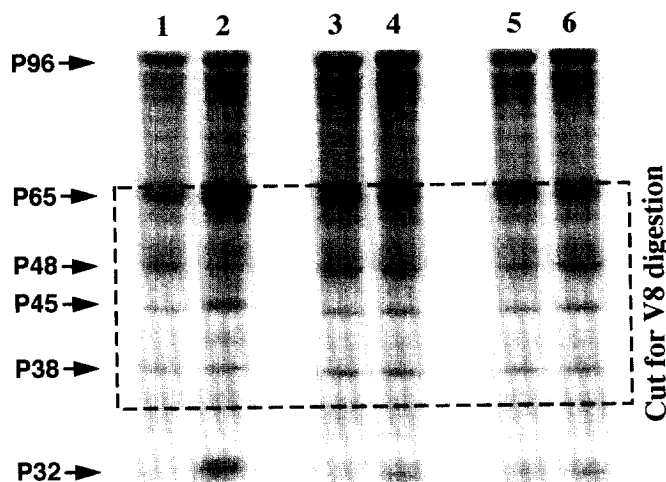*Values which are significantly different from control using two-tailed Student's t-test ($P<0.05$).

Fig. 1. Phosphorylation of proteins in intact chick ciliary ganglion. Intact ciliary ganglia were labelled with $^{32}P_i$ for 60 min and then treated for 10 min with control buffer (lane 1, 3 and 5), 2 μM PDBu (lane 2), 100 μM 8-Br-cAMP (lane 4) or 300 μM SNP (lane 6). Phosphoproteins were separated by 10% SDS–PAGE and visualized by autoradiography. The arrows indicate the location of phosphoproteins that showed consistent changes in phosphorylation. The dashed line shows the area which was cut out for the second dimension V8 protease digestion (Fig. 2). Results are representatives of at least three independent experiments.

endogenous substrates, activated under conditions that induced LTP in this system, we examined the pattern of protein phosphorylation obtained with freshly dissected intact ganglia following treatment with SNP or 8-Br-cAMP and compared it to the PDBu-induced phosphorylation changes. After pre-incubation of chick CG for 1 h in the presence of $^{32}P_i$ many proteins were phosphorylated under basal conditions (Fig. 1, lane 1, 3, 5; arrows) including phosphoproteins migrating at 96,000, 65,000, 48,000, 45,000 38,000 mol. wt and a triplet around 32,000 mol. wt (which will be referred to as P32). At this level of resolution the only proteins that showed consistently altered phosphorylation in all experiments after bath application of 2 μM PDBu were P65, P45 and P32 (Fig. 1, lane 2). In the presence of 100 μM cAMP (Fig. 1, lane 4) or 300 μM SNP (Fig. 1, lane 6) there were no major consistent changes in protein phosphorylation compared to basal levels. The other changes in the phosphorylation

visible in Fig. 1 were not observed in all experiments.

To investigate the phosphorylation changes at a higher level of resolution the region between 70,000 and 35,000 mol. wt (Fig. 1, dashed line) was cut from each gel track and segments from a control and a stimulated sample were applied to a second dimension 20% SDS–PAGE slab gel and the proteins were digested with V8 protease. Under our partial digestion conditions phosphoproteins at higher than 70,000 mol. wt were poorly digested by V8 protease therefore they were not included in this study. The phosphopeptides obtained were separated by electrophoresis. Under basal conditions (Fig. 2, Cont) the most heavily labelled peptides were P65a and b, P50a and b, P45a and b and a high molecular weight peptide labelled by an asterisk (*). The phosphorylation of peptides derived from P65 and P45 were all increased by the addition of PDBu (Fig. 2, PDBu) and to a small extent by 8-Br-cAMP (Fig. 2, cAMP)
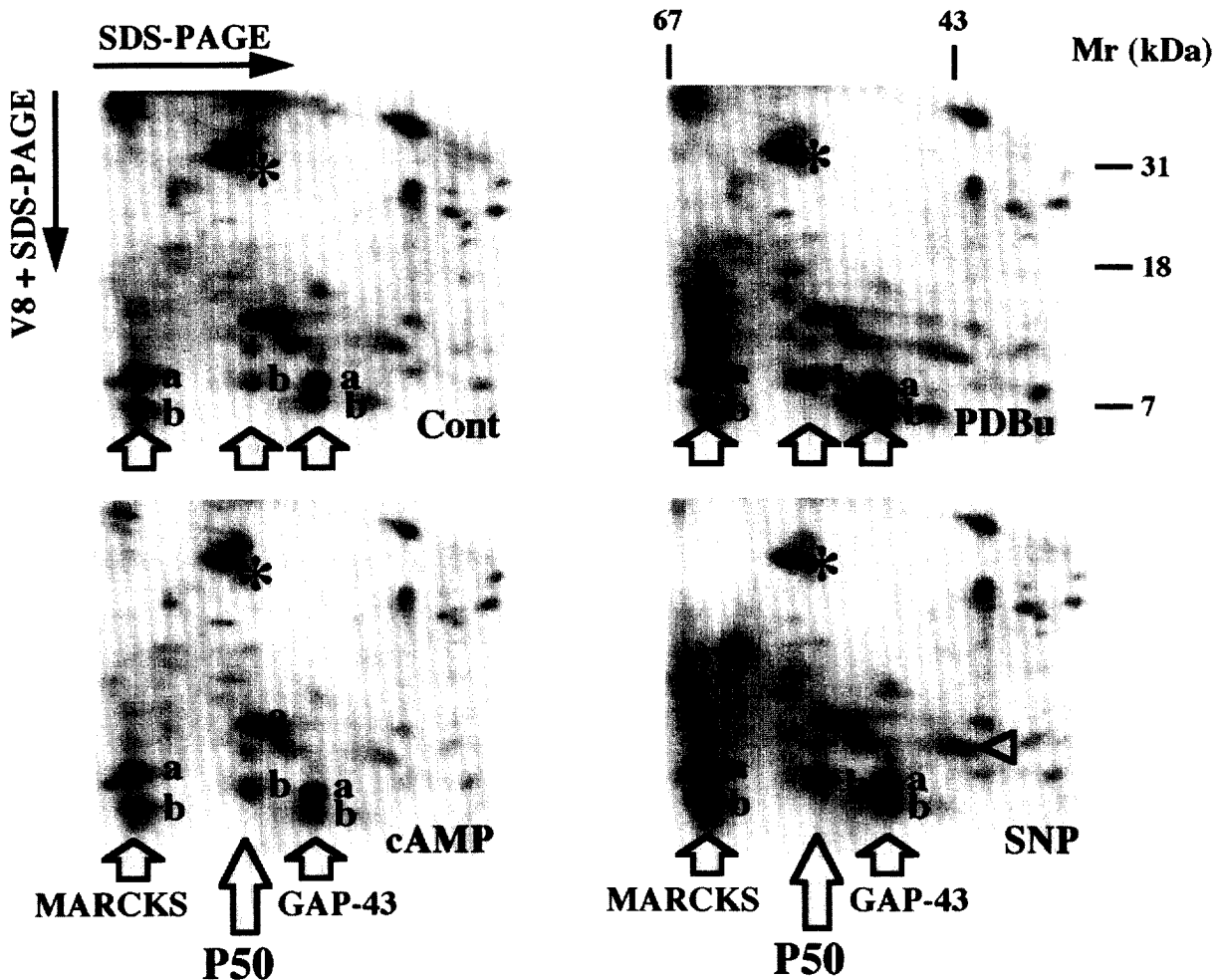
Fig. 2. Two-dimensional phosphopeptide patterns following V8 protease digestion. Intact CG were labelled with $^{32}P_i$ then treated as described in Fig. 1 and the 67,000–35,000 mol. wt region from 10% SDS–PAGE was cut out, rehydrated and digested by *Staphylococcus aureus* V8 protease in the stacking gel and separated on a second dimension 20% gel (V8+SDS–PAGE). Arrows indicate the position of phosphopeptides derived from MARCKS, GAP-43 or P50 (labelled with a and b next to the corresponding phosphopeptide); the open arrowhead indicates the phosphopeptide derived from P41 whose phosphorylation increased following SNP. An asterisk labels the phosphopeptide used to normalize data during quantitation by densitometry.

or, in certain experiments, by SNP (Fig. 2, SNP). There were three phosphopeptides derived from a protein(s) migrating at approximately 50,000 mol. wt. The apparent molecular weights of P50a and b were 15,000 and 11,000, respectively (Fig. 2). The phosphorylation of P50a and b was increased with application of 8-Br-cAMP and SNP, but did not change following the addition of PDBu. The phosphorylation of the third peptide, located between P50a and P50b, did not change reproducibly under any of the different incubation conditions so it will not be considered further. Note that P50a and P50b may be derived from different proteins since, in most experiments, P50a and P50b were not aligned in the second dimension. There were other phosphopeptides whose phosphorylation was increased with SNP, but their level of phosphorylation was so low

that accurate quantitation of these changes was not possible. One of the most clearly identifiable of these peptides whose phosphorylation was increased by SNP was P41 (Fig. 2, open arrowhead).

Since the phosphorylation of P65a and b was stimulated by PDBu and these peptides co-migrated with the [γ-^{32}P]ATP labelled and V8 digested peptides obtained after acid extraction of myristolated alanine-rich C kinase substrate (MARCKS) from CG homogenates[27] (results not shown) we tentatively identified P65 in intact CG as the MARCKS protein and P65a and b as the principal phosphopeptides produced from it by *Staphylococcus aureus* V8 protease. P45 was also acid extracted from intact $^{32}P_i$ labelled CG, its phosphorylation was stimulated by PDBu and it co-migrated in a 10% SDS–PAGE with [γ-^{32}P]ATP-labelled GAP-43 from CG
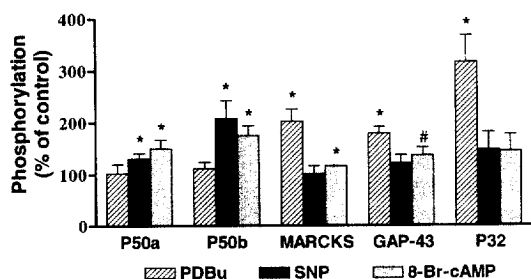
Fig. 3. Quantitation of the changes in the phosphorylation of selected V8-derived peptides in intact CG under the different incubation conditions. The phosphorylation of all the peptides derived from GAP-43 or MARCKS were combined for this figure. All values are expressed as percent of phosphorylation of the same peptide under basal conditions. The changes in the presence of 2 μM PDBu, 300 μM SNP or 100 μM 8-Br-cAMP are the averages of three to four experiments (± S.E.M.). Asterisk (*) labels the values which are significantly different from control using two-tailed Student's *t*-test ($P<0.05$) and # labels the value which is significantly different from control using one-tailed Student's *t*-test ($P<0.05$).

homogenates[27] and was therefore tentatively identified as GAP-43. The identity of P32 is not known, but a PKC substrate at the same molecular weight has been shown to be associated with synaptic membranes and to be a prominently-labelled phosphoprotein in intact rat brain synaptosomes (P. J. Robinson, unpublished observations).

The phosphorylation of the phosphopeptide labelled by the asterisk on Fig. 2 did not change in response to any of the activators used ($n=3$ for each condition; $P<0.05$, Student's *t*-test), but appeared to be dependent on the viability of the CG (data not shown). Because of the relatively constant labelling of this peptide we used its degree of labelling as an internal control for tissue viability and sample loading by normalizing the degree of phosphorylation of other peptides against this peptide. Following this normalization the change in phosphate incorporation into individual phosphopeptides was calculated by densitometry, taking the level of phosphorylation of the corresponding peptide under basal conditions as 100% (Fig. 3). Densitometric analysis and normalization allowed data from multiple experiments to be pooled ($n=3$, data represented as mean ± S.E.M.).

PDBu increased the phosphorylation of both MARCKSa and b as well as four other peptides of higher molecular weight that, based on a comparison of results from experiments with variable degrees of proteolytic digestion, appeared to be the result of incomplete digestion of MARCKS. For quantitation we pooled the optical density (OD) values of all six peptides. PDBu increased the phosphorylation of MARCKS-derived peptides by an average of $102 \pm 24\%$. The phosphorylation of MARCKS increased by $15 \pm 1.1\%$ in the presence of 8-Br-cAMP, while the addition of SNP caused no consistent change in the phosphorylation of these peptides.

Two phosphopeptides were obtained from neuromodulin (GAP-43) in intact CG: one which migrates faster than MARCKSa (GAP-43a) and the other which migrates slower than MARCKSb (GAP-43b). For quantitation we pooled the OD values of the two peptides. The phosphorylation of GAP-43 was increased by $79 \pm 13\%$ by PDBu and $36 \pm 15\%$ by 8-Br-cAMP, while SNP caused only an inconsistent change ($21 \pm 15\%$).

P32 was not digested by V8 protease under our limited digestion conditions and its phosphorylation was quantified from the first dimensional gels. Its phosphorylation was strongly stimulated by PDBu (average of $216 \pm 51\%$) and it was also stimulated in some experiments by SNP and 8-Br-cAMP, however the stimulation was highly variable and the increase was not statistically significant ($47 \pm 34\%$ and $45 \pm 32\%$, respectively; two-tailed Student's *t*-test; $P>0.05$).

While the phosphorylation of MARCKS, GAP-43 and P32 were all significantly increased the phosphorylation of P50a and b was not stimulated by PDBu. However, SNP and 8-Br-cAMP both stimulated the phosphorylation of P50a by $30 \pm 10\%$ and $50 \pm 16\%$, respectively and P50b by $108 \pm 34\%$ and $74 \pm 19\%$, respectively. The degree of stimulation of phosphorylation by SNP and 8-Br-cAMP were not significantly different from each other, but were significantly different from that induced by PDBu (two-tailed Student's *t*-test; $P<0.05$).

Ro31-8220, a cell permeable inhibitor of PKC, prevented the PDBu-induced phosphorylation of the peptides derived from MARCKS and GAP-43 (Fig. 4A; Ro31-8220+PDBu) and P32 (data not shown) in intact CG without affecting their basal phosphorylation (Fig. 4A). Ro31-8220 completely inhibited the SNP-induced phosphorylation of MARCKS and GAP-43 as well (data not shown), however it did not inhibit the SNP stimulated phosphorylation of P50a or P50b (Fig. 4B).

It was not possible to test whether the ability of SNP to induce LTP was blocked by Ro31-8220 because the application of Ro31-8220 resulted in a continuously drifting baseline in the electrophysiological recordings. This may be due to side effects of Ro31-8220 on acetylcholine receptors or other ion channels as reported in chromaffin cells.[32]

Cyclic GMP-dependent protein kinase and cyclic AMP-dependent protein kinase substrates in crude subcellular fractions from ciliary ganglion

To identify the enzyme(s) responsible for the phosphorylation of nitric oxide-responsive phosphoproteins (NORPs) subcellular fractions were prepared from frozen CG and phosphorylated *in vitro* in the presence of purified PKG, PKA or PKC, using the experimental procedure developed by Wang and Robinson.[57] In total homogenates under basal conditions the major phosphoproteins were P130, P53,
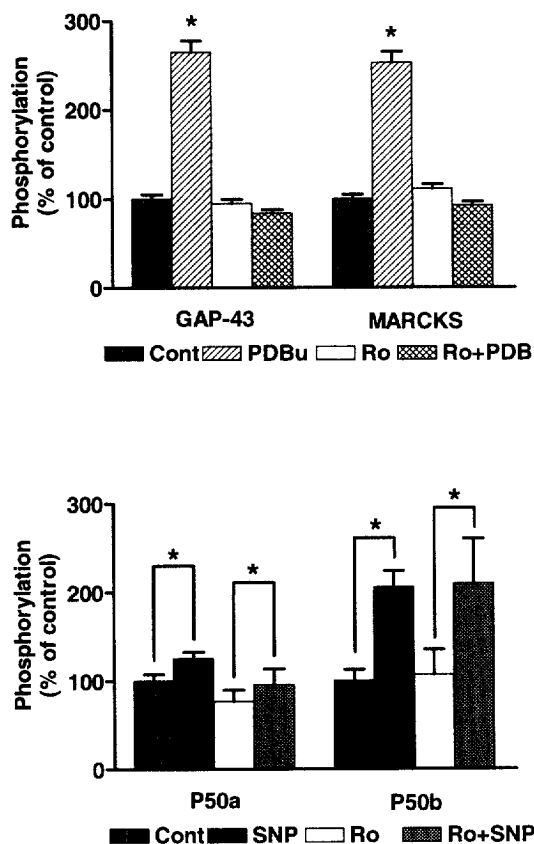
Fig. 4. Effect of PKC inhibitor Ro31-8220 on phosphorylation of key V8-derived peptides in chick CG. (A) Quantitative data for the phosphorylation of all the peptides from GAP-43 or MARCKS were combined for this figure. The PDBu-induced phosphorylation of GAP-43 or MARCKS was inhibited by Ro31-8220 (10 µM) while the basal phosphorylation was not affected. (B) SNP (300 µM) induced phosphorylation of P50a and P50b was not affected by Ro31-8220 (10 µM). Results are the averages of three experiments ± S.E.M. The asterisk indicates the values significantly different from the appropriate control values (two-tailed Student's t-test, $P<0.05$).

P43 and P32 (Fig. 5, lane 1). The addition of cGMP and PKG increased the phosphorylation of P43 and P130 (Fig. 5, lane 2). The open arrowhead indicates the autophosphorylated exogenous PKG (Fig. 5, open arrowhead). The addition of cAMP and PKA increased the phosphorylation of P40 and to a small extent P130 (Fig. 5, lane 3). The addition of Ca^{2+}/PS/DAG and PKC increased the phosphorylation of many proteins, including a group of high molecular weight proteins above 100,000, P96, P65, P61, a group of proteins between 53,000 and 50,000 mol. wt, P45, P38 and P32 (Fig. 5, lane 4).

Under basal conditions in the soluble fraction there were several proteins whose phosphorylation was not detected in total homogenates including P65, P61, P44 and P42. The phosphorylation of P32 was dramatically lower in the soluble fraction (Fig. 5, lane 5) while in the particulate fraction the phosphorylation of P32 was very high (Fig. 5, lane 9). There

were several phosphoproteins in the soluble fraction whose phosphorylation was increased by the addition of cGMP and PKG (P50, P45, P43, P42; Fig. 5, lane 6), but there were no visible PKG-induced changes in the particulate fraction (Fig. 5, lane 10). PKA increased the phosphorylation of several proteins including P50, P44, P43 and P40 in the soluble fraction (Fig. 5, lane 7), but it also caused no detectable changes in the phosphorylation pattern in the particulate fraction (Fig. 5, lane 11).

The addition of PKC to the subcellular fractions increased the phosphorylation of several proteins in the soluble fraction (P96, P65, P63, P45, P38 and P32; Fig. 5, lane 8 and 12), but caused only a small change in the phosphorylation of P65, P38 and P32 in the particulate fraction. The endogenous protein substrate(s) at 32,000 mol. wt are clearly present in both soluble and particulate fractions. However, the difference between the two fractions in the level of phosphorylation under basal conditions and the increase in phosphorylation after the addition of exogenous PKC suggest that the endogenous enzyme(s) responsible for the phosphorylation of P32 (possibly one of the endogenous PKC isoforms) is localized to the particulate fraction (Fig. 5, lane 12).

It is interesting to note that all the exogenous enzymes induced very little stimulation of phosphorylation in the particulate fraction. It is possible that in this fraction there is poor access to ATP or, as in case of P32, endogenous constitutively active kinases are enriched in this fraction causing high basal phosphorylation of substrates (compare control lanes 5 and 9 on Fig. 5).

Identification of nitric oxide-responsive phosphoproteins in subcellular fractions from ciliary ganglion

To investigate whether any of the PKG/PKA substrates identified in subcellular fractions of lysed CG may correspond to the NORPs in intact ganglia the V8 protease digestion was carried out on the 67,000 to 45,000 mol. wt (Fig. 6) and the 45,000–38,000 mol. wt regions (Fig. 7). Previous results have shown that the phosphopeptides derived from MARCKS, following $^{32}P_i$ labelling of intact tissue or *in vitro* [γ-^{32}P]ATP labelling of homogenized CG, co-migrate on 20% SDS–PAGE (data not shown). Therefore the positions of MARCKS a and b were used as internal references for the identification of NORP50a and b. The criteria for the identification of putative NORP50(s) *in vitro* were: (i) the phosphopeptides produced by V8 protease digestion from lysed tissue should migrate to the same relative position on the two-dimensional peptide map as NORP50a and b in intact CG, and (ii) they should be substrates for PKG and/or PKA, but not for PKC.

In total CG homogenate the phosphopeptide pattern obtained from the 67,000–45,000 mol. wt region (Fig. 6, Total) showed strong similarities to that of intact CG (Fig. 2). The two peptides derived from
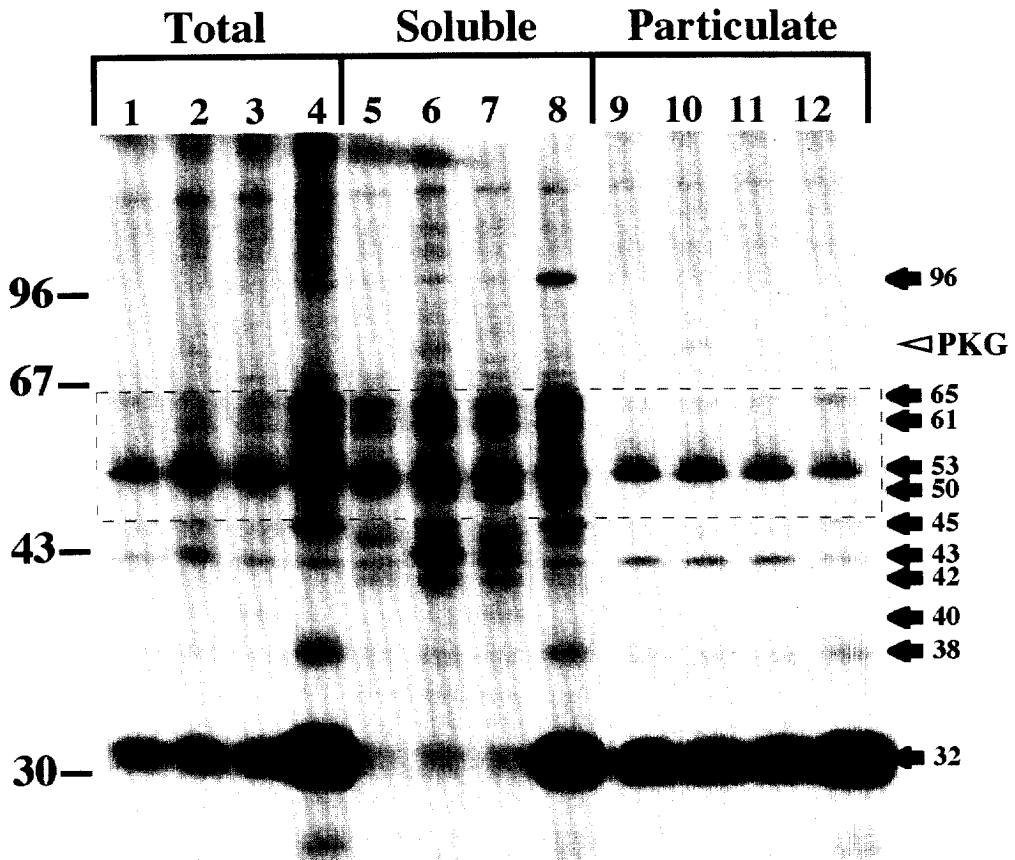
Fig. 5. Autoradiogram of protein phosphorylation in the total homogenate (1–4), the soluble fraction (5–8) and the particulate fraction (9–12) of ciliary ganglion. Ciliary ganglion extracts were phosphorylated with $[\gamma\text{-}^{32}P]ATP$ in the presence of EGTA/PKI (1, 5, 9), EGTA/cGMP/PKG/PKI (2, 6, 10), EGTA/cAMP/PKA (3, 7, 11) or Ca^{2+}/PS/DAG/PKC (4, 8, 12). Phosphoproteins were separated by 10% SDS–PAGE and visualized by autoradiography. The open arrowhead shows the position of exogenous purified PKG (75,000 mol. wt) while the closed arrowhead shows the position of exogenous purified catalytic subunit of PKA. The arrows indicate the position of key phosphoproteins whose apparent molecular weights are indicated in × 1000 mol.wt. The dashed line shows the area which was cut out for the second dimensional V8 protease digestion. The figure is a representative of three independent experiments.

proteins of approximately 50,000 mol. wt and marked a and b in Fig. 6 (Total), co-migrated in the second dimension with P50a and P50b from ^{32}P-labelled intact CG. However, it is important to note that the P50a and P50b phosphopeptides were clearly derived from proteins that had been well resolved in the first dimension gel, with the protein giving rise to P50a migrating with a smaller apparent molecular weight than the one giving rise to P50b (Fig. 6).

In the total homogenate the phosphorylation of P50a and P50b was stimulated by the addition of either PKG or PKA (Fig. 6). The addition of PKC increased the phosphorylation of many proteins that gave rise to phosphopeptides migrating in the region of interest, therefore it was not possible to determine whether the phosphorylation of P50a or P50b had been altered (Fig. 6, Total).

Following subcellular fractionation of the total homogenate P50b was detected in the particulate fraction while P50a was present in the soluble fraction (Fig. 6). This provides further evidence for the fact that these peptides are derived from different proteins. Both P50a and P50b were phosphorylated by PKG as well as PKA, but the addition of PKC, in this instance, decreased their phosphorylation (Fig. 6B).

Figure 7 shows a comparison of the phosphopeptide patterns obtained from proteins in the 45,000–38,000 mol. wt region after labelling intact ganglia with $^{32}P_i$ in the presence of SNP (Fig. 7A) and after labelling the soluble fraction from lysed tissue with $[\gamma\text{-}^{32}P]ATP$ in the presence of exogenous PKG (Fig. 7B). A protein at approximately 41,000 mol. wt gave rise to a peptide (labelled by a double arrowhead) whose phosphorylation was stimulated by both exogenous PKG (Fig. 7B) and PKA (not shown) *in vitro* and co-migrated with a peptide whose phosphorylation was stimulated *in vivo* by treatment of intact ganglia with SNP (Fig. 7A), but not by PDBu

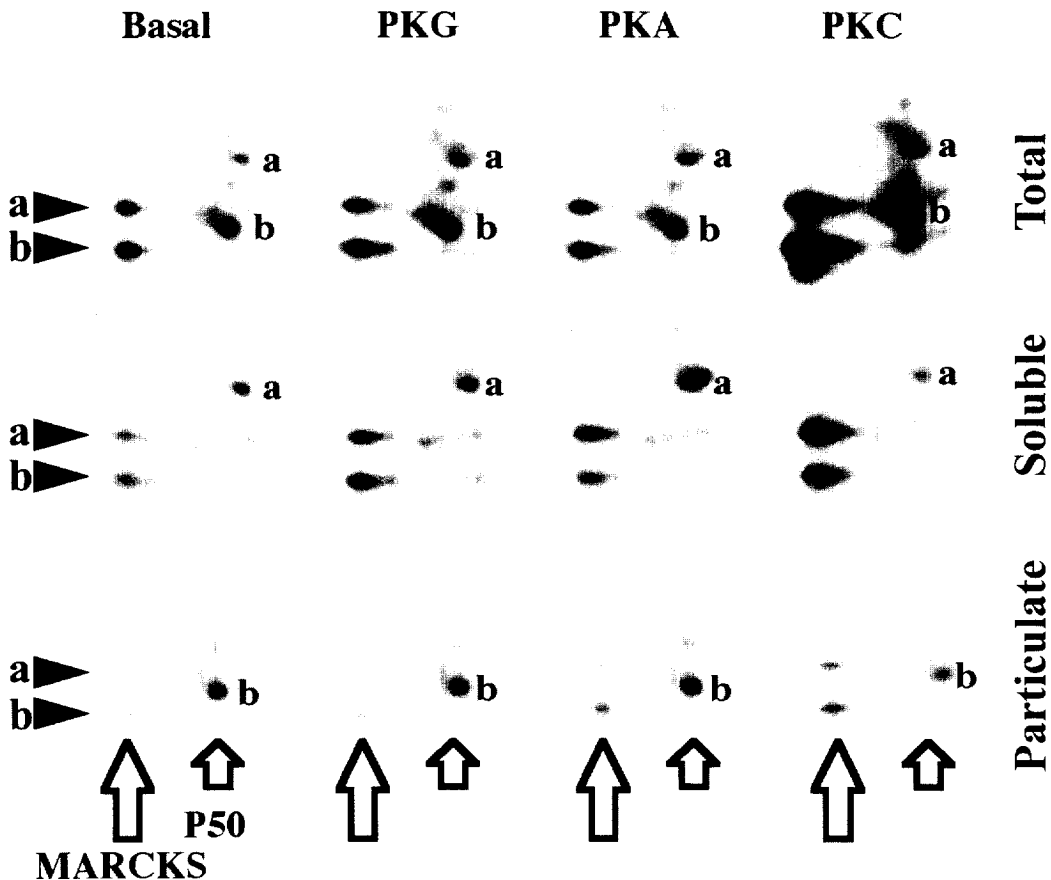**Basal**   **PKG**   **PKA**   **PKC**

Fig. 6. Two-dimensional phosphopeptide patterns of protein phosphorylation in subcellular fractions from CG. The subcellular fractions were treated as described in Fig. 5 and the 67,000–45,000 mol. wt region from the 10% gel was cut out, rehydrated and digested by *Staphylococcus aureus* V8 protease in the stacking gel and separated on a second dimension 20% gel. Arrowheads on the left show the position of the phosphopeptides derived from MARCKS which were used for the identification of P50a and P50b.

(Fig. 2). There were also a number of phosphopeptides that were substrates for PKG *in vitro*, but could not be identified in intact CG following incubation with SNP (Fig. 7, single open arrowheads).

DISCUSSION

The cholinergic synapses onto the ciliary neurons in the CG consist of a giant nerve terminal (calyx) with multiple release sites and display a presynaptically-induced form of LTP[48] that requires NO synthesis and the activation of protein kinases.[47] The induction of LTP requires Ca^{2+} influx into the presynaptic terminal,[48] but the locus of action of NO must be at a step(s) distal to Ca^{2+} entry as we have demonstrated that incubating CG with the NO donor SNP, under conditions known to induce LTP,[23,48] does not change the dynamics of either the calcium influx following an action potential or the basal level of intra-terminal calcium. Our previous work has shown that ciliary neurons and the calyces innervating them contain NOS[39] and have high basal levels of cGMP (L. Kang and M. R. Bennett, unpublished

observations) and that CG is enriched in PKG activity.[27] Therefore, we hypothesized that NO exerts its effect via activating guanylyl cyclase leading to an elevation of the levels of cGMP[37] and activation of PKG[12] in CG. This study provides direct evidence to support this proposal by identifying endogenous phosphoprotein substrates for PKG whose phosphorylation is increased by NO under conditions that induce LTP in CG.

Previous experiments have shown that bath application of SNP or 8-Br-cAMP to isolated intact ganglia induced LTP without the need for tetanic stimulation,[48] whereas bath application of PDBu did not.[47] We proposed that the molecular targets involved in LTP induction at these synapses will be endogenous substrates for PKG, perhaps also substrates for PKA, but not substrates for PKC. We labelled intact ganglia with $^{32}P_i$, treated them with SNP, 8Br-cAMP or PDBu, under conditions where the physiological consequences, in terms of LTP induction, were known, and then analysed the changes in protein phosphorylation. The most consistent responses to SNP treatment were shown by
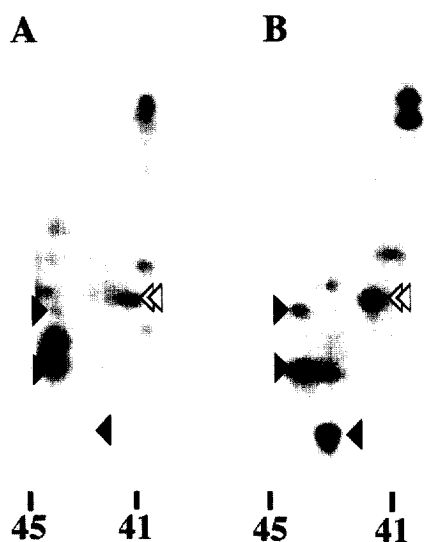
A   **B**

I   I   I   I
45   **41**   **45**   **41**

Fig. 7. Two-dimensional phosphopeptide patterns of phosphoproteins in the range between 45,000–38,000 mol. wt region following [32]P$_i$ labelling of intact CG (A) and [γ-^{32}P]ATP labelling of lysed cytosolic CG in the presence of cGMP/PKG (B). Double arrowhead shows the position of phosphopeptides whose phosphorylation was increased both in intact ganglia as well as lysed tissue. Single closed arrowheads label the peptides derived from proteins whose phosphorylation increased only *in vitro.*

the phosphopeptides P50a and P50b, which were derived from proteins of approximately 50,000 mol. wt and a phosphopeptide P41. The phosphorylation of these three NORPs was also stimulated by 8-Br-cAMP, but not by PDBu. Because of the uncertainty of the concentration of intracellular 8-Br-cAMP achieved throughout the incubation of intact CG it was not clear from these experiments whether the effect of 8-Br-cAMP was due to direct activation of PKA or cross-activation of PKG.[21]

To identify the kinase(s) responsible for the phosphorylation of NORPs in intact tissue we incubated total homogenates and subcellular fractions from CG with purified PKG, PKA and PKC using the approach of Wang and Robinson.[57] This technique allowed us to identify several previously undetected PKG substrates in CG homogenates.[27] We also identified PKG substrates whose phosphopeptides appeared to co-migrate with P50a, P50b and P41, the phosphopeptides derived from NORPs after [32]P$_i$ labelling of intact CG. These PKG substrates were also phosphorylated by PKA, but not by PKC, therefore we postulate that these phosphoproteins detected in lysed tissue correspond to NORPs from intact tissue. Separation of the soluble and particulate proteins from homogenized CG showed that the NORPs are localized to different subcellular compartments. The proteins that gave rise to P50a and P41 were both present in the soluble fraction whereas the protein that gave rise to P50b, the phosphopeptide that gave the largest SNP-induced increase

in phosphorylation, was localized to the particulate fraction. The identification of NORPs provides an additional component for the NO/cGMP/PKG-mediated signal transduction pathway in CG. These results also show that NORPs may also be a point of convergence for PKG and PKA-dependent signalling pathways in CG.

The NO/cGMP pathway is important in intracellular signalling in both the peripheral and the CNS.[13,46] PKG-mediated protein phosphorylation is one of the possible mechanisms through which this pathway can exert its effects. However, there are not many well-characterized endogenous phosphoprotein substrates for PKG in the nervous system and even fewer whose phosphorylation is changed when NO releasing agents are applied.[58] The only exception is DARPP-32, a dopamine and cAMP-regulated phosphoprotein of mol. wt 32,000, whose phosphorylation is stimulated by SNP[55] in the substantia nigra, a neuronal system in which the presence of the full signalling pathway from NOS to the phosphorylation of a PKG substrate (DARPP-32) has been demonstrated.[2,55] We did not find any evidence for the phosphorylation of DARPP-32 in CG. The triplet at mol. wt 32,000, that we refer to as P32, did not appear to be DARPP-32 since the proteins that comprise P32 are PKC substrates that are resistant to *Staphylococcus aureus* V8 protease digestion, whereas DARPP-32 is a substrate for PKA and PKG[16] and is digested by V8 protease.[53] There is indirect evidence suggesting that the NO/cGMP/PKG pathway is involved in the regulation of the phosphorylation of certain ion channels[6,15,51] and receptors,[25,42,59] however, the phosphorylation of these receptors and ion channels by this pathway *in vivo* remains to be demonstrated. Because of their cytosolic localization, P50a and P41 are not likely to be receptors or ion channels. Whether the membrane associated P50b identified in this study is a receptor or an ion channels, has yet to be determined. The existence of soluble as well as membrane-associated NORPs could be especially relevant in the view of the recent results which showed that not all forms of NOS play a role in LTP in hippocampal pyramidal neurons, but only the membrane-associated endothelial NOS.[22,40] Therefore NORPs localized to different subcellular compartment might play differential roles in LTP in CG.

Exposure to PDBu resulted in an increase in the phosphorylation of several proteins in intact CG. The most heavily labelled of these were MARCKS, GAP-43 and P32. Following the addition of either SNP or 8-Br-cAMP there was a small increase in the phosphorylation of these proteins, which was blocked by the addition of Ro31-8220, showing that it was due to PKC activation. The phosphorylation of these substrates is most likely to occur via cross-activation of PKC by PKG and/or PKA. Such cross-talk has been proposed in several systems based on results obtained with purified enzymes,[14] lysed

tissue[27] and PC12 cells,[60] but, to our knowledge, this is the first evidence that it exists in an intact neuronal tissue. The functional significance of this cross-talk for synaptic plasticity in CG is not known, but, since the strong activation of PKC by PDBu was not able to induce LTP,[47] this weak effect on PKC substrates by SNP or 8-Br-cAMP is either not involved in, or not sufficient to induce, LTP in CG. In hippocampus, the phosphorylation of GAP-43 has been shown to increase following tetanic stimulation,[41] but not by application of NO,[29] despite the fact that NO has been shown to be involved in the induction of LTP in hippocampus.[17] This suggests that in the hippocampus, as in the CG, NO exerts its effect via a pathway which does not require PKC activation.

CONCLUSION

The discovery of three NORPs, in addition to the previously described DARPP-32, significantly extends our understanding of NO signalling in neurons. Which, if any, of the NORPs identified so far may be specifically involved in the induction of LTP in CG remains to be established. The characterization of these NORPs provides new directions for the investigation of the effects of NO in many parts of the nervous system.

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