cAMP[7]. As AA and its cytochrome P_{450} metabolites are potent inhibitors of ($Na^+ + K^+$) ATPase[17], their formation may account for the synergism described in striatal neurons.

We have shown here that D_2 agonists enhanced Ca^{2+}-stimulated AA release at nM concentrations and with EC_{50} 20- to 30-fold lower than the corresponding K_i for receptor binding[13]. This suggests that partial D_2 receptor occupation is sufficient to produce a robust AA response. It will be important to determine whether this facilitatory action, described here for transfected CHO cells, occurs in brain areas, such as the striatum, where comparable D_2 receptor densities are found and receptor-stimulated AA release has been demonstrated[18].

The facilitatory effect of D_2 receptors on Ca^{2+}-dependent AA release reported here suggests that, in presynaptic terminals, the Ca^{2+} increase which follows depolarization and triggers dopamine release may, at the same time, enable D_2 autoreceptors[19] to release AA and its metabolites. These may, in turn, participate in the autoinhibitory actions of dopamine, for example by modulating K^+ or Ca^{2+} channel activities[20-23] or protein phosphorylation[24]. Finally, as costimulation of a Ca^{2+}-mobilizing receptor revealed an unexpected amplification by dopamine of AA release, more neurotransmitters than previously thought may operate through the AA cascade by a similar mechanism.

Note added in proof: A study by Kanterman *et al.*[29] has appeared after submission of the present study, showing that stimulation of D_2 receptors in transfected CHO cells augments A23187-induced [^{3}H]AA release. □

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A role for peptide in determining MHC class II structure

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T LYMPHOCYTES recognize antigen-derived peptides associated with major histocompatibility complex (MHC) class I or class II proteins[1,2]. Peptide is critical in class I heavy-chain folding and/or stable association with β_2-microglobulin[3-6]. Although data exist suggesting a relationship between class II structure and peptide association[7-9], no equivalent positive contribution of peptide to the folding state or stability of class II dimers has yet been demonstrated. We report here that most purified $E\alpha^kE\beta^k$ molecules leaving low pH in the absence of specific peptide lack a compact, stable dimeric structure. Brief exposure to the appropriate peptide just before and during neutralization promotes this specific conformation in proportion to stably bound peptide, indicating that peptide is important in determining class II MHC structure. Our results also indicate that efficient generation of long-lived peptide-class II complexes involves two stages: initial peptide binding in an acidic environment, which enhances the ability of class II to enter a conformation, from which stabilization upon neutralization results in high-affinity binding of previously associated peptide.

When analysed without reduction or boiling, class II molecules show three distinct forms on electrophoresis in SDS-polyacrylamide gels[8]: 'compact' (C) dimers migrating with an apparent relative molecular mass of 56,000 (M_r, 56K), 'floppy' (F) dimers (63-67K) which are partially denatured C dimers, and disassembled α (31-33K) and β (28-30K) chains. To investigate the relationship between peptide binding and the structure of class II, we isolated $E\alpha^kE\beta^k$ molecules[7] and analysed their gel migration under a variety of conditions. Most purified $E\alpha^kE\beta^k$ incubated at pH 7.2 with or without added peptide migrates as C dimers of 56K; a small fraction (5-10%) migrates as free α and β chains (Fig. 1*a* and *b*). Incubation for 30 min at pH 4.5 and 37 °C, followed by neutralization, converts most of the C dimers into either F dimers or free α and β chains. These α and β chains derive from SDS dissociation of 'unstable' (U) dimers, based on co-precipitation by anti-C terminal peptide antisera (data not shown). The presence during acid treatment and neutralization of any of several peptides known to bind to $E\alpha^kE\beta^k$, leads to retention of the C structure and a concomitant diminution in F or U dimers (Fig. 1*a* and *b*). For most $E\alpha^kE\beta^k$ to retain the C form, 50-100 μM peptide is required; 10 μM peptide has barely detectable effects (results not shown). Activity is related to binding to $E\alpha^kE\beta^k$, as peptides lacking this property (hen egg lysozyme (HEL) residues 46-61, interacts with $A\alpha^kA\beta^k$ (ref. 10); ovalbumen

TABLE 1 Specificity of the peptide effect on $E\alpha^kE\beta^k$ conformation

$E\alpha^kE\beta^k$ incubation conditions	Relative protein density*			
	Floppy	Compact	$E\alpha$	$E\beta$
pH 4.5, no peptide → pH 7.2	49.0	19.0	8.0	5.0
pH 4.5 + DASP → pH 7.2	23.0	48.0	2.0	3.0
pH 4.5 + OVA 323-339 → pH 7.2	60.0	14.0	4.0	6.0
pH 4.5 + HEL 46-61 → pH 7.2	48.0	20.0	7.0	3.0

* SDS-polyacrylamide gel stained with silver was scanned for protein density. Results for each $E\alpha^kE\beta^k$ form are expressed as per cent of total protein in the applied sample, as determined by laser densitometry. Less than 25% of total $E\alpha^kE\beta^k$ appears as high molecular weight bands (>90K) in every sample. Experimental conditions for treatment and gel analysis are as described in the legend to Fig. 1*b*. Peptides were used at 100 μM. All peptides were shown to have appropriate bioactivity by stimulation of T-cell hybridomas in the context $E\alpha^kE\beta^k$ (DASP), $A\alpha^dA\beta^d$(OVA 323-339), and $A\alpha^kA\beta^k$ (HEL 46-61).

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(OVA) residues 323-339, interacts with $A\alpha^dA\beta^d$ (refs 11, 12)) do not prevent loss of the C structure (Table 1).

We used western blotting to investigate high-affinity (slow dissociation rate) peptide binding. $E\alpha^kE\beta^k$ incubated at acid pH, then neutralized in the presence of biotinylated des-Ala-splice cytochrome peptide (DASP), remains associated with peptide throughout SDS-PAGE and western transfer. The strong peptide signal displayed at the C dimer position (Fig. 2*a*) correlates with efficient generation of the C form as determined by silver stain (Fig. 2*b*). Additional strong peptide signals at 90K and 110K, that have been previously observed, are present[13]; the chain composition of these forms is unknown, but they are distinct from F dimers (Fig. 2*a*, lane 4). $E\alpha^kE\beta^k$ incubated for 2 hours with peptide at neutral pH binds 17-fold less peptide, and no change in the amount of C dimer is detectable. Samples that are acid-treated then neutralized before DASP addition also show little peptide binding or C dimer (Fig. 2*a*). Therefore, generation of additional empty binding sites during low-pH treatment does not fully account for the marked enhancement of peptide binding observed in these experiments, as has been found using a functional assay[14].

The apparent exchange of endogenous peptides for added DASP at pH 4.5, and the requirement for high (50-100 μM) peptide concentrations to maximally preserve the C state, are most consistent with an equilibrium reaction of low affinity (rapid dissociation rate) at acidic pH. However, differences between the pH required for dissociation of endogenous peptides[15,16] versus binding of DASP could also explain these findings. To examine this issue, we pre-loaded $E\alpha^kE\beta^k$ molecules with biotinylated DASP, then exposed them to pH 4.5 for varying periods of time. Only 19% of pre-bound peptide remains bound to C-forms after incubation without additional peptide for 1.5 h at pH 4.5 and 37 °C (Fig. 3). Addition of 100 μM free biotinylated peptide 5 min before neutralization, results in a peptide signal similar to that of samples maintained at neutral pH. Compact dimers decrease from 85% to 15% of total class II if the preparation is exposed to low pH and neutralized in the absence of additional peptide, whereas C dimers totalling 44% of total protein are present if peptide is added. Longer (3.5 h) incubation at low pH leads to >95% loss of previously bound peptide and there is substantial binding of added peptide, which reaches 47% of the original level. A lower level of C molecules is achieved (13% without peptide, 27% with peptide at 3.5 h). Thus, enhanced dissociation and effective binding of the same peptide are both observed at pH 4.5. The interactions of peptide and class II required for promoting the C state occur rapidly at pH 4.5, with nearly half the maximum possible effect being seen within only 5 min of peptide-class II interaction. Further, the amount of C form and the extent of high-affinity peptide binding are directly proportional under these conditions.

The unusual kinetics of stable peptide binding to class II at neutral pH (refs 7, 17), the multiple discrete conformers of class II showing differential peptide association[8,9,18], the capacity of low pH to enhance peptide-class II interactions[14,15], and the role of acidic vesicles in antigen processing[19], all led us to investigate whether peptide plays a substantial part in the folding state/stability of class II molecules achieved following transit out of an acidic environment. Our results provide direct evidence

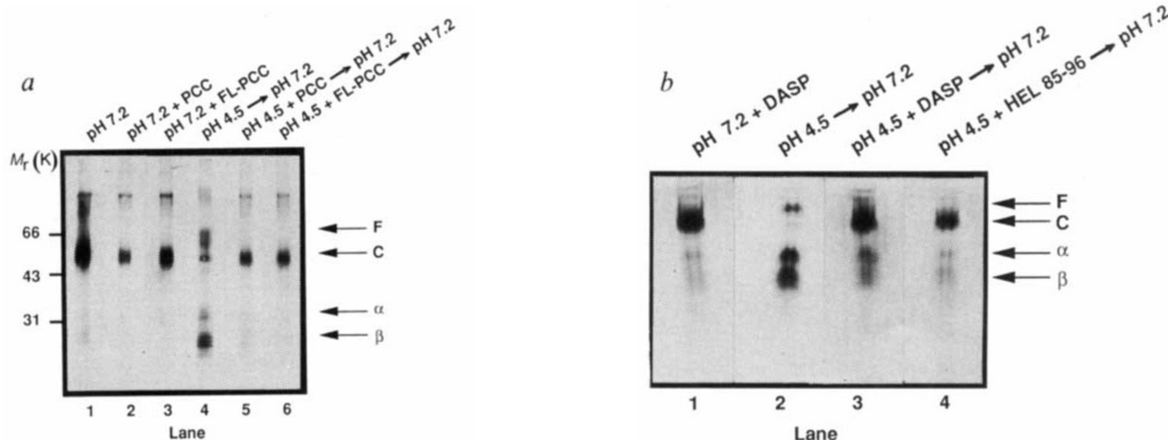

FIG. 1 The presence or absence of specific peptide determines the conformation of $E\alpha^kE\beta^k$ molecules leaving an acidic environment. *a*, Silver-stained SDS-polyacrylamide of $E\alpha^kE\beta^k$ in lipid vesicles: lane 1, incubated at pH 7.2 without added peptide; lane 2, incubated at pH 7.2 with pigeon cytochrome *c* (PCC) 88-104; lane 3, incubated at pH 7.2 with fluorescein-labelled PCC(FL-PCC) 88-104; lane 4, incubated at pH 4.5, then brought to pH 7.2 in the absence of peptide; lane 5, incubated at pH 4.5 with PCC 88-104, then brought to pH 7.2; lane 6, incubated at pH 4.5 with FL-PCC 88-104, then brought to pH 7.2. Numbers on the left of the figure represent molecular weight standards; F, floppy $E\alpha^kE\beta^k$ dimers (63-67K); C, compact stable $E\alpha^kE\beta^k$ dimers (56K); α, 33K α chain derived from U dimers dissociated in SDS buffer; and β, 27K β chain derived from U dimers dissociated in SDS buffer. Protein bands appear to be wide because of glycosylation of MHC and unreduced disulphide bonds present in MHC structure. *b*, Silver-stained SDS-polyacrylamide gel of $E\alpha^kE\beta^k$ in detergent micelles: lane 1, incubated at pH 7.2 with DASP; lane 2, incubated at pH 4.5, then brought to pH 7.2 without adding peptide; lane 3, incubated at pH 4.5 with DASP, then brought to pH 7.2; lane 4, incubated at pH 4.5 with HEL 85-96, then brought to pH 7.2.
METHODS. $E\alpha^kE\beta^k$ was purified from lipopolysaccharide-stimulated CBA/J B cell blasts using monoclonal antibody 14-4-4 S (ref. 23) for affinity chromatography as described[7]. Purified $E\alpha^kE\beta^k$ was stored in 30 mM octylglucoside in Tris (100 mM), NaCl (140 mM), sodium azide (0.02%) (OG/Tris) at pH 8.3, 4 °C. For the experiment shown in Fig. 1*a*, the purified class II molecules were inserted in lipid vesicles as previously described[7]. For the experiment shown in Fig. 1*b*, the purified class II molecules in OG/Tris were used directly. Aliquots of 50 μl of 1 μM $E\alpha^kE\beta^k$ at neutral pH (7.2) were either incubated at 37 °C without further additions or with a final concentration of 100 μM peptide. Identical aliquots were brought to pH 4.5 by adding 0.4 μl of 2.0 M acetic acid and incubated at 37 °C either without further additions or with 100 μM peptide added at the same time as the acid. After 30 min, the pH of the samples was readjusted to 7.2 by adding 0.4 μl of 1 M Tris, and the neutralized samples incubated for an additional 2-3 h at 37 °C. Later experiments have shown that this additional post-neutralization incubation is unnecessary for the effects reported here (see Fig. 3). Each sample (10 μl) was added to 25 μl sample buffer[24] without 2-mercaptoethanol, and loaded without prior boiling on a 12% linear polyacrylamide gel to permit assessment of the conformation/stability of the $E\alpha^kE\beta^k$ dimers. Gels were run at 200 V for ~45 min then silver stained[25]. The effective pH for induction of unfolding and successful refolding was between 4.5-5, depending on such variables as whether $E\alpha^kE\beta^k$ was inserted into lipid vesicles or assayed in detergent micelles, and how soon after preparation the class II protein was tested. All peptides were prepared by solid phase synthesis and HPLC-purified before use. Sequence of PCC 88-104 is KAERADLIAYLKQATAK (one-letter amino-acid code); FL-PCC is PCC 88-104 with a fluorescein group on the amino terminus—this is a separate synthesis of the core PCC peptide; HEL 85-96, SSDITASVNCAK; DASP, KKANELIAYLKQATK.

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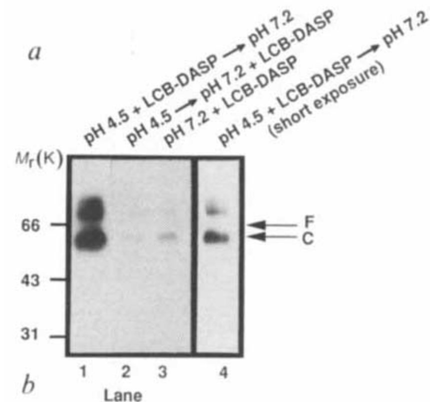

	Protein stain			
F	18	18	28	Per cent of total protein
C	35	15	36	
α	12	15	9	
β	14	25	6	

FIG. 2 Peptide is most efficient in binding to $E\alpha^kE\beta^k$ and promoting the compact conformation if offered during low-pH transit. *a*, Western blot for bound peptide. Lane 1, $E\alpha^kE\beta^k$ incubated at pH 4.5 with biotinylated DASP (LCB-DASP), then brought to pH 7.2; lane 2, $E\alpha^kE\beta^k$ incubated at pH 4.5 without added peptide, brought to pH 7.2, then incubated with biotinylated DASP; lane 3, $E\alpha^kE\beta^k$ incubated at pH 7.2 with biotinylated DASP; lane 4, a lighter exposure of lane 1. *b*, Numbers are derived from a parallel silver-stained gel and represent the per cent of each conformation over total stained protein per lane.

METHODS. $E\alpha^kE\beta^k$ in OG/Tris was treated as described in the legend to Fig. 1 with the following changes: the peptide used was DASP conjugated on the amino terminus according to ref. 26. For detection of biotinylated peptide bound to $E\alpha^kE\beta^k$, MHC-peptide complexes were transferred from the SDS-polyacrylamide gel to nitrocellulose membranes (Hybond) for 1.5 h at 100 V in 20 mM Tris/150 mM glycine, pH 8.3. Biotinylated peptide associated with transferred proteins was detected with horseradish peroxidase-conjugated avidin and chemiluminescence (Enhanced Chemiluminescence Kit, Amersham). The protein distribution on silver-stained gels and the protein-bound biotinylated peptide signal revealed by chemiluminescence were measured by laser scanning densitometry using a Shimadzu CS-9000.

for an important positive contribution of peptide to the C structure of typical class II molecules, and indicate that this form is associated with the most stable peptide-binding state. Striking differences have been reported in the pH required for optimal binding of certain peptides versus that necessary for dissociation of complexes of the same peptide-class II pair already formed[15]. This is consistent with a significant effect of peptide on class II binding site conformation, an effect we find reflected in alterations in affinity for certain monoclonal antibodies (S.S.N. and R.N.G., unpublished observations). Our observations of changes in class II structure during intracellular maturation and transport[20] are fully consistent with our *in vitro* data using purified class II free of invariant chain. These metabolic labelling studies showed an antigen-dependent conversion of U to C dimers in a post-Golgi compartment following invariant chain dissociation, the same change as observed here for acid-treated mature molecules stripped of endogenous peptide and re-exposed to exogenous peptide during neutralization.

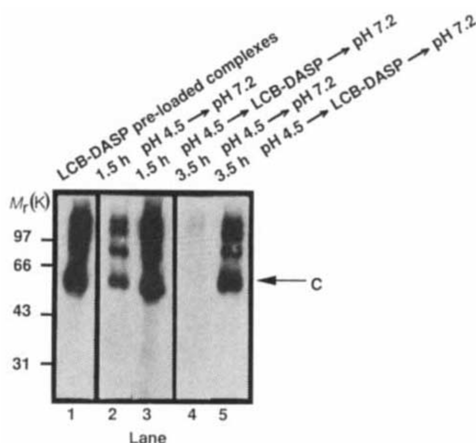

FIG. 3 Dissociation of biotinylated peptide from $E\alpha^kE\beta^k$ under acidic conditions and fast binding of peptide to empty molecules. Western blot showing bound peptide. Lane 1, $E\alpha^kE\beta^k$ molecules pre-loaded with biotinylated-DASP (LCB-DASP); lane 2, pre-loaded peptide: $E\alpha^kE\beta^k$ complexes incubated for 1.5 h at pH 4.5 without added peptide, then neutralized; lane 3, preloaded peptide: $E\alpha^kE\beta^k$ complexes incubated for 1.5 h at pH 4.5, incubated for 5 min with biotinylated DASP, then brought to pH 7.2; lane 4, same as lane 2, but incubated at pH 4.5 for 3.5 h; lane 5, same as lane 3, but incubated at pH 4.5 for 3.5 h before peptide addition.

METHODS. $E\alpha^kE\beta^k$: biotinylated DASP complexes were prepared by incubating 1 μM $E\alpha^kE\beta^k$ with 100 μM biotinylated DASP at neutral pH. $E\alpha^kE\beta^k$ containing a significant fraction of biotinylated DASP: $E\alpha^kE\beta^k$ complexes prepared in this manner were separated from free peptide using Millipore Ultrafree-MC filter units with a 10K cut-off point. This $E\alpha^kE\beta^k$ containing pre-bound biotinylated DASP was incubated at pH 4.5 and 37 °C for the times indicated and then neutralized either in the absence of additional peptide (lanes 2 and 4) or presence of 100 μM biotinylated DASP peptide added 5 min before neutralization (lanes 3 and 5). Samples were immediately subjected to SDS-PAGE, one gel was then silver-stained, and a duplicate used for western blotting as described in the legend to Fig. 2.

It seems paradoxical that the same pH can enhance both dissociation of previously bound peptide and entry of added peptide into stable complexes. $E\alpha^kE\beta^k$ forms short-lived but specific peptide complexes at neutral pH that slowly convert to long-lived forms associated with the C state[7]. These data may indicate a rigidity of class II at neutral pH that only rarely permits conformational change of the binding region, retarding both formation and dissociation of C complexes. At low pH, the class II molecule may gain more flexibility, facilitating exit from the C state and loss of previously bound peptide. Added peptide could then enter available sites and efficiently drive the system towards, if not fully into, the C state. Additional class II stabilization upon emergence from the acidic environment would then give rise to typical long-lived complexes. This model, similar to one already proposed[21], implies that peptide acquisition by class II in an acid compartment, followed by emergence into a neutral environment, results in the 'trapping' of processed antigen as stable complexes. This may contribute to the ability of class II to present peptides with a low effective affinity in the acidic environment where initial interaction may take place. The apparent absence of a similar stabilizing mechanism for class I-peptide complexes may limit this limb of the antigen presentation system to peptides with a high intrinsic affinity for class I, perhaps explaining observations suggesting a smaller useful number of determinants in various proteins for class I versus class II-dependent T-cell stimulation[22]. □

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Dissection of the protein kinase cascade by which nerve growth factor activates MAP kinases

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MITOGEN activated protein (MAP) kinases (MAPKs) are a family of protein-serine/threonine kinases activated as an early intracellular response to a variety of hormones and growth factors[1–4]. They are unique in requiring both serine/threonine and tyrosine phosphorylation to become active[5] and are the only examples of protein-serine/threonine kinases activated by tyrosine phosphorylation. Nerve growth factor (NGF) promotes differentiation of phaeochromocytoma (PC12) cells, which respond by conversion within hours from a chromaffin-like to a sympathetic neuron-like phenotype[6,7]. NGF stimulation of PC12 cells increases the activity of two protein kinases by >20-fold within minutes[8], both strikingly similar to MAPKs. They are inactivated by either protein-tyrosine phosphatases or the protein-serine/threonine phosphatase termed protein phosphatase 2A (ref. 8), they activate protein S6 kinase-II (refs 9, 10), and they phosphorylate identical threonine residues on myelin basic protein (our unpublished results) to those phosphorylated by other MAPKs[11,12]. Immunological data[13] indicate that these protein kinases, termed peak-I and peak-II (Fig. 1*a*) are probably ERK2 and ERK1, respectively, two widely expressed MAPK isoforms[13]. Here we identify the 'MAP kinase kinases' (MAPKKs) in PC12 cells which are activated by NGF and report that MAPKKs are dependent on serine/threonine phosphorylation for activity and promote phosphorylation of serine/threonine and tyrosine residues on MAPKs.

We screened the fractions from Mono Q in Fig. 1*a* for activities able to reactivate the peak-I MAPK previously inactivated by incubation with either CD45 (a protein-tyrosine phosphatase (PTPase)) or protein phosphatase 2A (PP2A). These experiments revealed Mg-ATP-dependent reactivating activity using either substrate, that was confined to fractions eluting just before and overlapping with the peak-I MAPK (Fig. 1*a*) and which is hereafter referred to as MAPKK. MAPKK activity was pooled (Fig. 1*a*) and subjected to successive chromatographies on Mono S, Mono Q and Superose 12 (Fig. 1). Identical profiles were obtained whether MAPKK was assayed with CD45-treated MAPK ('MAP(Tyr)KK') or PP2A-treated MAPK ('MAP(Ser/Thr)KK'). MAPKK was resolved into two peaks on Mono Q (Fig. 1*c*), both possessing the same MAP(Tyr)KK: MAP(Ser/Thr)KK activity ratio. Each eluted from Superose as proteins of relative molecular mass 50,000–55,000 (M_r 50–55K) (Fig. 1*d*). If NaCl was omitted, MAP(Tyr)KK and MAP(Ser/Thr)KK again coeluted, but with an apparent M_r of 40K (not shown).

Extracts from unstimulated cells contain inactive MAPK, which elutes from Mono Q at the position of active peak-I MAPK (Fig. 2*a*). Inactive MAPK was reactivated by purified MAPKK using [γ-^{32}P]ATP, and rechromatography resolved two active components (Fig. 2*c*) coeluting with the peak-I and peak-II MAPKs. Thus inactive MAPK in unstimulated cells (Fig. 2*a*) is the precursor of both active MAPK isoforms, and MAPKK can activate each species. The ^{32}P-labelled MAPKs migrated as 42K (peak-I) and 44K (peak-II) proteins (Fig. 2*c* inset), consistent with their identity as ERK2 and ERK1, respectively[13]. Both the peak I (Fig. 2*d*) and peak II (not shown) isoforms were phosphorylated on serine/threonine and tyrosine, establishing that MAPKK phosphorylates both types of phosphoamino acid. Incubation with PTPases inactivated peak I and II by >90%, released 30% of the ^{32}P radioactivity (not shown) and dephosphorylated tyrosine specifically (Fig. 2*d*), whereas incubation with PP2A also inactivated peaks I and II by >90%, released 70% of the ^{32}P radioactivity (not shown), and dephosphorylated serine and threonine specifically (Fig. 2*d*). No MAPKK activity was detectable when extracts from unstimulated cells were chromatographed on Mono Q (Fig. 2*b*), demonstrating that activation of MAPKK requires NGF stimulation.

MAP(Tyr)KK and MAP(Ser/Thr)KK were both inactivated by preincubation with PP2A (Fig. 3*a, b*). Inactivation was blocked by the PP2A inhibitor okadaic acid[14], but unaffected by orthovanadate (0.2 mM), a general PTPase inhibitor, indicating that PP2A did not activate contaminating PTPase which then inactivated MAPKK. Consistent with dephosphorylation of serine/threonine residues, three PTPases (CD45 (ref. 15), LAR (ref. 16) and T-Cell phosphatase (ref. 17)) had no effect on either MAP(Tyr)KK or MAP(Ser/Thr)KK (Fig. 3*a, b*) under conditions where they inactivated MAPK by 80% (not shown). Although PP2A dephosphorylates some tyrosine residues in the presence of divalent cations[18], inactivation of MAPKK by PP2A does not seem to involve tyrosine dephosphorylation because inactivation occurs without divalent cations. In addition preincubating PP2A with 0.2 mM pyrophosphate or 1 mM ATP (which inactivates its protein serine/threonine phosphatase activity while enhancing its PTPase activity[18]) prevents inactivation of MAPKK (not shown).

MAPK inactivated by either CD45 or PP2A shows no reactivation on subsequent incubation with Mg-ATP, demonstrating that neither the tyrosine nor the serine/threonine residues are phosphorylated at significant rates by MAPK itself, and suggesting that MAPKK is a protein kinase. But an interaction may occur between MAPKK and MAPK inducing conformational changes that enhance enormously the rate of autophosphorylation of MAPK. This idea was raised by Ahn *et al.*[19], who recently identified two MAPK activators in epidermal growth factor (EGF)-stimulated 3T3 cells. These resemble our MAPKKs because they reactivate MAPK inactivated by either CD45 or PP2A (ref. 19).

MAPK is activated by tyrosine phosphorylation and stimulated by NGF and other growth factors whose receptors are protein-tyrosine kinases[1,2,20]. Consequently, there had been optimism that very few intervening steps might exist between receptor and MAPK activation. This hope is dashed by our results, which indicate that MAPKK is activated by serine/threonine phosphorylation, implying that 'MAP kinase kinase kinase' is a protein-serine/threonine kinase (Fig. 4). But one can now propose how okadaic acid and phorbol esters, which inhibit protein-serine/threonine phosphatases and activate protein kinase C (a serine/threonine kinase), respectively, might cause tyrosine phosphorylation and activation of MAPKs (refs 2, 21). These tumour promoters may stimulate serine/threonine phosphorylation of MAPKK.

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