Tyrosine kinase inhibitors enhance a Ca^{2+}-activated K^+ current (I_{AHP}) and reduce I_{AHP} suppression by a metabotropic glutamate receptor agonist in rat dentate granule neurones

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1. Activation of metabotropic glutamate receptors (mGluRs) inhibits a transient Ca^{2+}-activated K^+ current (I_{AHP}) responsible for the slow after-hyperpolarization that follows depolarizations of dentate granule neurones in rat hippocampal brain slices. Here we show for the first time that this physiological consequence of mGluR stimulation is selectively attenuated by blockers of protein tyrosine kinases (PTKs).

2. Several distinct types of PTK blockers, including genistein, tyrphostin-B42 and lavendustin-A, reduced the inhibition of I_{AHP} by the selective mGluR agonist (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD). Inhibition of I_{AHP} by 5-HT was unaffected. The PTK blockers by themselves doubled the duration of I_{AHP} suggesting that there exists a tonic inhibitory influence on I_{AHP} that is reduced by PTK antagonists.

3. Inclusion of EGTA (1 mM) in the patch pipette also potentiated the I_{AHP} and reduced the inhibitory action of ACPD on I_{AHP}, consistent with the observation of others that chelation of intracellular Ca^{2+} prevents protein tyrosine phosphorylation induced by ACPD.

4. We propose that mGluR-initiated inositol 1,4,5-trisphosphate ($InsP_3$) production mobilizes intracellular Ca^{2+} and leads to increased protein tyrosine phosphorylation which in turn leads to inhibition of I_{AHP}.

Tyrosine kinase-mediated phosphorylation of proteins has been recognized as an important mechanism for controlling gene expression and the consequent regulation of cell proliferation, growth and development (Schlessinger & Ulrich, 1992). Biochemical and immunohistochemical studies have shown widespread distribution of protein tyrosine kinases (PTKs) in the brain with particularly high expression in the hippocampus (see Sanchez, Tapley, Saini, He, Pulido & Barbacid, 1994; Lev *et al.* 1995). The abundance of these enzymes in mature, non-dividing cells such as neurones suggests their involvement in the moment-to-moment regulation of physiological processes, distinct from their involvement in the control of gene expression. This suggestion is supported by the ability of specific PTK antagonists to disrupt a number of physiological responses (see Levitsky, 1992). Protein tyrosine phosphorylation, which reflects a balance between PTK and tyrosine phosphatase activity, is known to be involved in the modulation of ion channel activity. Increased protein tyrosine phosphorylation leads to enhancement of NMDA-mediated currents in cultured spinal cord neurones while PTK antagonists reduce those currents (Wang & Salter, 1994). Tyrosine phosphatases influence the gating mode of a cation channel in *Aplysia* bag cells and the responsiveness of those currents to protein kinase A (Wilson & Kaczmarek, 1993) and tyrosine phosphorylation of a delayed rectifier current $I_{K(V)1.2}$ contributes to the Ca^{2+}-dependent inhibition of that current (Huang, Morielli & Peralta, 1993).

These molecular consequences of protein tyrosine phosphorylation may have implications for neuronal integration and neuropathology: PTK inhibitors block the induction of long term potentiation (LTP) of hippocampal synaptic transmission (O'Dell, Kandel & Grant, 1991) and reduce delayed neuronal death induced by hypoxia (Kindy, 1993). Blockade of metabotropic glutamate receptors (mGluRs) often have similar actions to PTK antagonists. Induction of LTP has been reported to be disrupted by antagonists of

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mGluRs (Bortolotto, Bashir, Davies & Collingridge, 1994; but see Brown & Reymann, 1995 for negative results in dentate granule neurones) and neurotoxicity is potentiated by a selective mGluR agonist (McDonald & Schoepp, 1992). Recently, activation of mGluRs has been shown to increase tyrosine phosphorylation of several proteins in hippocampal slices and hippocampal neurones in culture (Siciliano, Gelman & Girault, 1994) raising the possibility that certain consequences of mGluR activation may be mediated by second messenger cascades dependent upon tyrosine phosphorylation.

I_{AHP} is a Ca^{2+}-activated K^+ current activated by Ca^{2+} influx during depolarization of central nervous system neurones. Following repolarization and termination of Ca^{2+} influx, the current decays over a period of a few seconds as Ca^{2+} is cleared from the neurone. This current underlies the slow after-hyperpolarization (AHP) observed in many neurones following a depolarization and provides an intrinsic inhibitory influence that plays a pivotal role in regulating neuronal excitability. As such I_{AHP} is an important target of neuromodulation by neurotransmitters. Neurotransmitter-mediated inhibition at I_{AHP} has been linked to activation of a variety of kinases. However, the non-selective kinase antagonist staurosporine has no effect on inhibition produced by the selective mGluR agonist (1*S*,3*R*)-1-amino-cyclopentane-1,3-dicarboxylic acid (ACPD). This is true even when protein kinase A (PKA)- and protein kinase C (PKC)-mediated suppression of I_{AHP} in the same cell is clearly blocked (see Abdul-Ghani, Valiante, Carlen & Pennefather, 1996). Here we show that inhibition of I_{AHP} produced by ACPD in dentate granule neurones of rat hippocampal brain slices is reduced by known inhibitors of tyrosine kinases. These effects are seen at concentrations of PTK antagonists that have no affect on the action of 5-HT, a neurotransmitter that inhibits I_{AHP} by activating adenylate cyclase and PKA, arguing against a non-specific action of these PTK blockers (see Abdul-Ghani *et al.* 1996).

METHODS

The methods used were essentially the same as those described elsewhere (Abdul-Ghani *et al.* 1996). Briefly, transverse brain slices (400 μm thick) were obtained from male Wistar rats (150–180 g) after anaesthesia with halothane and decapitation. The slices were maintained in artificial cerebrospinal fluid (ACSF) composed of (mM): 125 NaCl, 5 KCl, 1·25 NaH_2PO_4, 25 $NaHCO_3$, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose; osmolarity was 300 mosmol kg^{-1}. The pH was set at 7·4 and held by bubbling with 95% O_2–5% CO_2 and adding HCl. When recording I_{AHP} the ACSF was supplemented with a number of agents to block the K^+ and Na^+ currents. These agents were (mM): 5 tetraethylammonium, 2,4-aminopyridine, 2 $CsCl_2$, 0·0005 TTX. For whole-cell recording the pipette-filling solution contained (mM): 150 potassium methylsulphate, 10 Hepes, 2 MgATP with pH adjusted to 7·2 with KOH; osmolarity was 280 mosmol kg^{-1}. The PTK blockers genistein, tyrphostin-B42 and lavendustin-A as well as diadzein, the inactive analogue of genistein, were first dissolved in a DMSO stock solution and then diluted in recording solution before use to 0·1% or less. Concentrations of DMSO up to 0·5% did not interfere with ACPD inhibition of I_{AHP}. In order to economize on drugs and reduce depletion due to solubility problems and non-specific binding, the PTK inhibitors were often applied intracellularly by including the blocker in the pipette-filling solution. With extracellular application, the preparation was exposed to the blockers for at least 10 min to ensure equilibration before addition of ACPD. Intra-pipette tyrphostin appeared to interfere with seal formation. Accordingly, with tyrphostin the electrode was first tip filled with standard internal solution and then back filled with the same electrode solution with the addition of tyrphostin. After establishing whole-cell recording, 30 min was allowed for equilibration before applying ACPD. All values are presented as means ± S.E.M. ACPD was obtained from Tocris Neuramin (Bristol, UK). The tyrosine kinase antagonist and analogues were obtained from Calbiochem.

RESULTS

ACPD (10 μM) caused a rapid, complete and reversible inhibition of I_{AHP} (Fig. 1*A*). Bath application of genistein, at a concentration of 100 μM, which has been shown to be specific in inhibiting hippocampal PTKs without influencing the activity of other protein kinases in brain slices (O'Dell *et al.* 1991), reduced the inhibition of I_{AHP} by ACPD from 95 ± 5% ($n = 12$) to only 23 ± 5% ($n = 7$) (Fig. 1*B*, Table 1). This effect of the inhibitor suggests that PTK activity contributes to the action of mGluRs in inhibiting I_{AHP}. Genistein also enhanced I_{AHP} and prolonged its decay time (Fig. 1*B*). Such a potentiating action would be expected if genistein had an action opposite to that of ACPD, namely increasing rather than decreasing the sensitivity of AHP channels to Ca^{2+}. In five cells in which the effect was tested, 100 μM genistein increased I_{AHP} area by 237 ± 62%, and caused a 260 ± 70% increase in the duration of decay from the I_{AHP} peak to 70% of that peak. The potentiating effect of genistein on I_{AHP} suggests that there is tonic activity of the PTKs involved in the inhibition of I_{AHP} produced by activation of mGluRs. Evidence for tonic PTK or PTK-dependent activity has recently been presented for rat spinal cord neurones, where PTK antagonists reduce currents activated by NMDA receptors (Wang & Salter, 1994). However, this tonic activation is not due to activation of mGluR receptors; the non-selective mGluR antagonist *R,S*-α-methyl-4-carboxyphenylglycine (MCPG) causes only a small (16%) enhancement of I_{AHP} (Abdul-Ghani *et al.* 1996). Table 1 shows that in the presence of the PTK antagonists, the onset of the suppression of I_{AHP} by ACPD is several times slower than normal. This indicates that despite the potentiation of I_{AHP} by the PTK antagonists the remaining inhibition of I_{AHP} by ACPD differs fundamentally from that normally observed and in such a way that is consistent with inhibition of the ACPD action by PTK antagonists.

Daidzein is a structural analogue of genistein that does not inhibit PTKs. It had no effect on either the size of the I_{AHP} or its time course and did not affect the ability of ACPD to

Table 1. Summary of the effects of tyrosine kinase inhibitors and analogues on the inhibitory action of ACPD on I_{AHP} in dentate granule neurones

Condition	n	Inhibition of I_{AHP} by 10 μM ACPD[1] (%)	Time from peak to 70% of peak[2] (s)	Time constant ACPD action[3] (s)
Control	12	95 ± 5	1·6 ± 0·1	39 ± 5
Diadzein (100 μM)	3	88 ± 10	1·8 ± 0·3	30 ± 6
Genistein (100 μM)	7	23 ± 5*	3·9 ± 0·2*	379 ± 39*
Lavendustin-A (500 μM)[4]	7	52 ± 8*	2·9 ± 0·2*	200 ± 20*
Tyrphostin-B42 (500 μM)[4]	3	44 ± 4*	3·0 ± 0·2*	150 ± 9*
EGTA (1 mM)[4]	7	43 ± 8*	3·2 ± 0·1*	165 ± 11*

I_{AHP} was evoked every 30 s and measured in terms of charge transfer. The time constant of inhibition was estimated from an exponential fit to the fractional inhibition of successive currents recorded after beginning the application of ACPD. *$P < 0·001$, compared with control values. [1] Measured in terms of reduction of the integral of the first 6000 ms of I_{AHP}. [2] Peak is maximal outward current 100–1000 ms after the end of the depolarizing step used to activate I_{AHP}. [3] Time constant of onset determined by fitting an exponential function to the first 5–6 points (e.g. 25 s) after beginning superfusion of ACPD. [4] Intracellular application of kinase inhibitor.

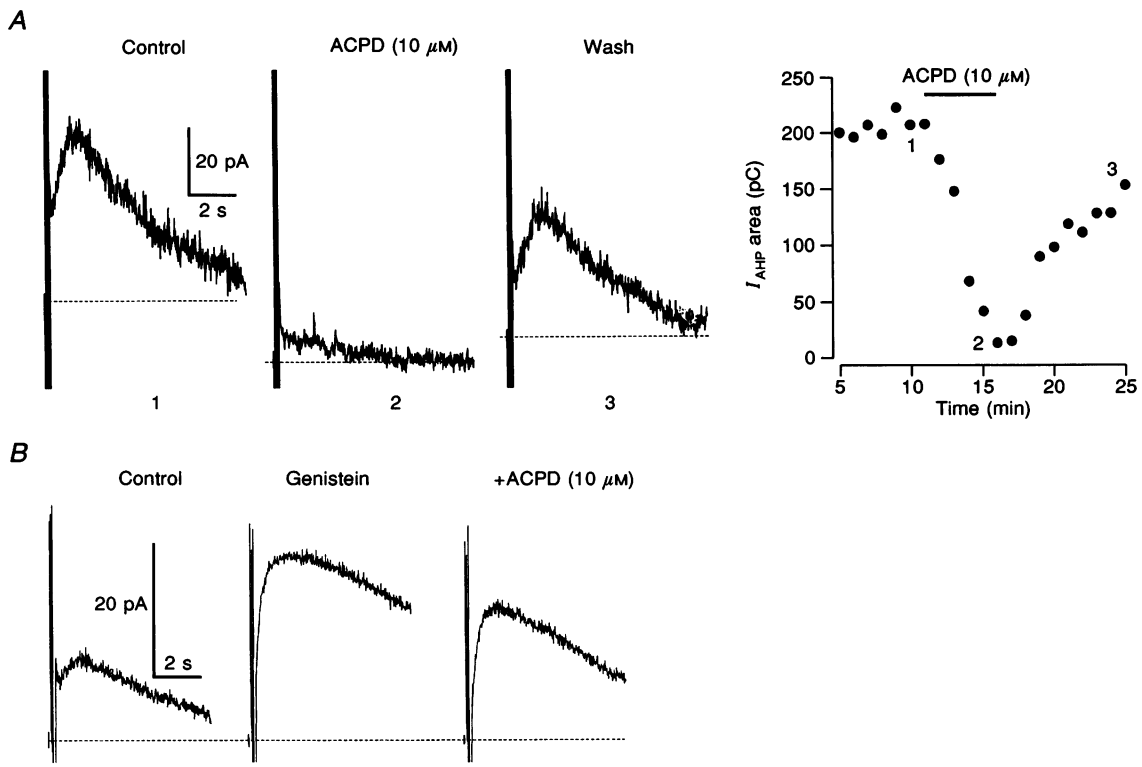

Figure 1. Inhibition of I_{AHP} by ACPD and attenuation of this action by genistein, a selective PTK inhibitor

A, ACPD (10 μM) caused a rapid, complete and mostly reversible reduction of I_{AHP}. The numbers below current traces correspond to time points indicated on the graph depicting the time course of ACPD action. *B*, extracellular application of genistein (100 μM) increased the size of I_{AHP} and prolonged its duration; it also reduced the ability of ACPD to block it.

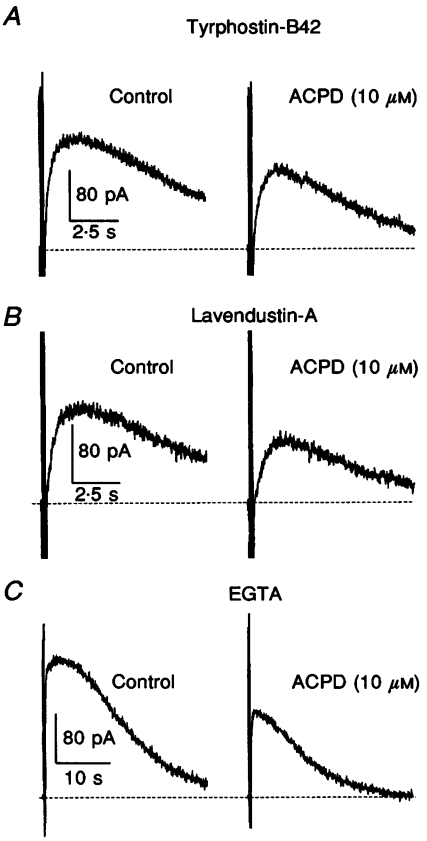

Figure 2. Other tyrosine kinase inhibitors block ACPD inhibition of I_{AHP}

A, intracellular dialysis with 500 μM tyrphostin-B42 reduced the ACPD inhibition of I_{AHP}. *B*, intracellular dialysis with 500 μM lavendustin-A was also effective in blocking inhibition of I_{AHP} by ACPD. *C*, intracellular dialysis with 1 mM EGTA mimicked the action of specific PTK blockers. Note that these PTK inhibitors by themselves prolonged the duration of I_{AHP} (compare with control I_{AHP} in Fig. 1*A* and *B*; see Table 1).

inhibit I_{AHP} (Table 1). Members of other classes of PTK inhibitors that differ from genistein also attenuated the action of ACPD. Intracellular dialysis with either tyrphostin-B42 (Levitski, 1992) or lavendustin-A (O'Dell *et al.* 1991) reduced the inhibitory action of ACPD on I_{AHP} (Fig. 2; Table 1). Thus, antagonism of the action of ACPD by genistein is dependent on its ability to inhibit PTK activity. Like genistein, tyrphostin and lavendustin also caused a prolongation of I_{AHP} (Table 1) consistent with a tonic PTK mediated influence on the current.

Tyrosine phosphorylation of proteins induced by ACPD in brain slices is prevented by interfering with the ability of ACPD to raise intracellular Ca^{2+} levels by loading neurones with a membrane-permeable Ca^{2+} chelator (see Siciliano *et al.* 1994). Thus, Ca^{2+} chelators can be considered as physiological antagonists of the PTKs involved in mGluR activity. Indeed, inclusion of the Ca^{2+} chelator EGTA in the patch pipette filling solution inhibits the action of ACPD (Fig. 2*C*; Table 1; see also Abdul-Ghani *et al.* 1996). Here we show that the action of EGTA on I_{AHP} resembles in many ways the action of tyrosine kinase inhibitors (Fig. 2*C*); the

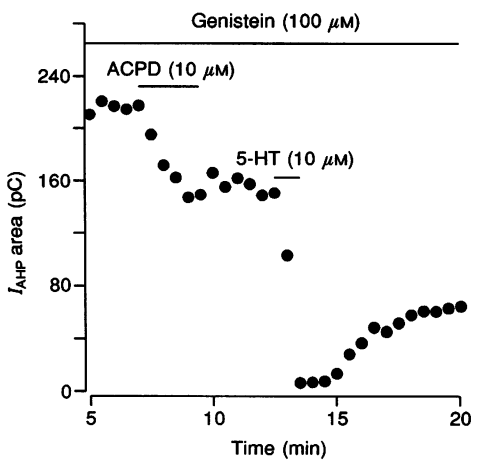

Figure 3. Intracellular dialysis with genistein (10This reduces inhibition of I_{AHP} by ACPD, but does not influence the inhibition of I_{AHP} by 5-HT (10 μM). Note that intracellular delivery of genistein was as effective as bath application (see Table 1) at reducing the inhibition of I_{AHP}.

initial decay rate of I_{AHP} is slowed, as is the rate of development of ACPD blockade, and the level of ACPD blockade is reduced (see Table 1). These results are consistent with the hypothesis that the process that regulates I_{AHP} and is blocked by the PTK antagonist is also Ca^{2+} dependent.

The blocking effect of genistein appears to be specific to the second messenger cascade that couples mGluRs to inhibition of I_{AHP}. Genistein did not interfere with the second messenger cascade that leads to inhibition of I_{AHP} by 5-HT (Fig. 3*A*), a neurotransmitter that inhibits I_{AHP} through activation of adenylate cyclase and PKA (see Abdul-Ghani *et al.* 1996), indicating that genistein is interfering selectively with the coupling of mGluRs to this current.

DISCUSSION

To the best of our knowledge, this report is the first to demonstrate that a physiological action of mGluRs can be modulated by antagonists of PTK activity. These results also suggest for the first time that PTK antagonists, and by implication, phosphorylation of protein tyrosine residues in dentate granule neurones of the hippocampus, modulate the activity of I_{AHP}, an important intrinsic inhibitory current in CNS neurones. The fact that three structurally unrelated inhibitors of PTKs interfered with ACPD action while daidzein, the structural analogue of genistein that is unable to block the enzyme, did not strongly suggests that the effects observed in this study are indeed due to inhibition of PTKs. Of course, a definitive validation of this hypothesis will require a careful pharmacological study that quantitatively correlates inhibition of tyrosine phosphorylation with inhibition of the ability of ACPD to suppress I_{AHP}. Our results nevertheless indicate that the effect of the PTK inhibitors is specific to the inhibition of I_{AHP} by ACPD since inhibition of I_{AHP} by 5-HT was unchanged.

How the targeted PTKs fit into the second messenger cascade leading to I_{AHP} inhibition is not yet clear. Of the eight types of mGluRs cloned and identified so far, none appears to be directly coupled to the activation of PTKs and all have the seven transmembrane domains typical of G protein-coupled receptors (Pin & Duvoissin, 1995). We have presented evidence elsewhere (Abdul-Ghani *et al.* 1996) that ACPD-mediated inhibition of I_{AHP} involves G protein-dependent production of inositol trisphosphate ($InsP_3$) and the consequent mobilization of Ca^{2+} from intracellular stores. The action is blocked by heparin and neomycin and mediated by a pharmacological subtype of mGluR known to be coupled to $InsP_3$ breakdown. G protein activation of $InsP_3$ production involves stimulation of phospholipase-β (PLC-β) which, unlike PLC-γ, is generally not thought to be dependent on protein tyrosine phosphorylation.

It is known that certain tyrosine residues in $InsP_3$ receptors can be phosphorylated (Harnick, Jayaraman, Ma, Mulieri, Go & Marks, 1995). Thus it is possible that $InsP_3$ action is compromized by the PTK antagonists. There is also one report of tyrphostins interfering with the production of $InsP_3$ (in pancreatic acinar cells in response to carbachol and cholecystokinin octapeptide (Piiper *et al.* 1994)). Thus, it is possible that protein tyrosine phosphorylation influences the efficacy of mGluR-mediated activation of $InsP_3$ production. Although we cannot rule out this mechanism, the fact that PTK antagonists potentiate I_{AHP} in the absence of mGluR activation and influence the time course of ACPD action in a way that differs from simply reducing ACPD concentration suggests to us that the PTK antagonists act downstream from the mGluR in the second messenger cascade initiated by ACPD. It is our opinion that a likely mechanism linking mGluR stimulation, $InsP_3$ production, PTK activation and I_{AHP} inhibition involves Ca^{2+}. Several studies have shown that activation of mGluRs by ACPD elevates intracellular calcium levels presumably through mobilization of Ca^{2+} from $InsP_3$-sensitive intracellular stores (see Pin & Duvoisin, 1995). There is evidence for interactions between phosphoinositide turnover, Ca^{2+} mobilization and tyrosine phosphorylation (Huckle, Dy & Earp, 1992; Lee, Toscas & Villereal, 1993; Huang *et al.* 1993; Lev *et al.* 1995) and that, induction of protein tyrosine phosphorylation by a number of stimuli is Ca^{2+} dependent (Stratton, Worley, Huganir & Baraban, 1989; Huckle *et al.* 1992; Huang *et al.* 1993; Siciliano *et al.* 1994; Lev *et al.* 1995). It seems reasonable, therefore, to suggest that the protein tyrosine phosphorylation-dependent step is downstream from release of $InsP_3$ and Ca^{2+} mobilization in the second messenger cascade initiated by mGluR activation. The similarities between the actions of EGTA and PTK blockers (see Fig. 2) are also consistent with this hypothesis. However, the hypothesis implies paradoxically that elevation of cytosolic Ca^{2+} can lead to both activation and inhibition of I_{AHP}.

Since the time course of the Ca^{2+} signal responsible for generating I_{AHP} is unchanged during inhibition by ACPD (see Charpak, Gahwiler, Do & Knopfel, 1990), it seems likely that ACPD and ACPD-induced protein tyrosine phosphorylation could affect the interpretation of that signal by the Ca^{2+}-activated K^+ channel (decreased Ca^{2+} affinity) rather than modifying the handling of Ca^{2+} by the cell (see Fig. 1*C*). Other factors that may be relevant are differences in time course and subcellular localization between Ca^{2+} signals responsible for I_{AHP} generation and inhibition. The intracellular Ca^{2+} signal responsible for I_{AHP} reaches a peak within a few hundred milliseconds and decays to baseline over a few seconds, while the action of ACPD to inhibit I_{AHP} develops over 1 min and once developed does not appear to be associated with an obvious macroscopic elevation of intracellular Ca^{2+} (see Charpak *et al.* 1990). It is nevertheless possible that localized changes in Ca^{2+} levels or transient changes in those levels are sufficient to activate Ca^{2+}-dependent tyrosine phosphorylation. Along these lines, it is notable that Ca^{2+} released from $InsP_3$ stores may be more effective at increasing tyrosine phosphorylation then a generalized elevation of intracellular Ca^{2+} levels (see Meucci,

Scorziello, Avallone, Florio & Schettini, 1995). Indeed, some reports have suggested that the balance between tyrosine phosphorylation and dephosphorylation can be influenced by the level of filling of $InsP_3$-sensitive Ca^{2+} stores, such that depletion of those stores leads to increased levels of protein tyrosine phosphorylation (Sargent, Frandale & Sage, 1994; Meucci *et al.* 1995).

Regardless of the exact mechanism involved, a link between stimulation of mGluRs and activation of protein tyrosine phosphorylation may have implications for understanding the mechanisms responsible for other physiological and pathophysiological processes dependent on activation of both mGluRs and protein tyrosine phosphorylation such as LTP (O'Dell *et al.* 1991; Bortolotto *et al.* 1994) and delayed neuronal death (Kindy, 1992; McDonald & Shoepp, 1992). Inhibition of the action of ACPD on I_{AHP} by PTK blockers may provide a window on the activity of a physiologically important protein tyrosine phosphorylation system that may also be involved in the induction of LTP.

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