Receptor protein tyrosine phosphatase α participates in the m1 muscarinic acetylcholine receptor-dependent regulation of Kv1.2 channel activity

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The phosphorylation state of a given tyrosine residue is determined by both protein tyrosine kinase (PTK) and protein tyrosine phosphatase (PTP) activities. However, little is known about the functional interaction of these opposing activities at the level of an identified effector molecule. G protein-coupled receptors (GPCRs), including the m1 muscarinic acetylcholine receptor (mAChR), regulate a tyrosine kinase activity that phosphorylates and suppresses current generated by the Kv1.2 potassium channel. We examined the possibility that PTPs also participate in this signaling pathway since the tyrosine phosphatase inhibitor vanadate increases the extent of both Kv1.2 phosphorylation and suppression. We show that an endogenous transmembrane tyrosine phosphatase, receptor tyrosine phosphatase α (RPTP α), becomes tyrosine phosphorylated and co-immunoprecipitates with Kv1.2 in a manner dependent on m1 receptor activation. The N- and C-termini of Kv1.2 are shown to bind RPTP α *in vitro*. Overexpression of RPTP α in *Xenopus* oocytes increases resting Kv1.2 current. Biochemical and electrophysiological analysis reveals that recruiting RPTP α to Kv1.2 functionally reverses the tyrosine kinase-induced phosphorylation and suppression of Kv1.2 current in mammalian cells. Taken together, these results identify RPTP α as a new target of m1 mAChR signaling and reveal a novel regulatory mechanism whereby GPCR-mediated suppression of a potassium channel depends on the coordinate and parallel regulation of PTK and PTP activities.

Keywords: G proteins/muscarinic receptors/potassium channels/receptor tyrosine phosphatases

Introduction

Reversible tyrosine phosphorylation is a common mechanism by which protein activity is regulated. The coordinate action of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) sets the steady-state phosphorylation level of a given protein. While the importance of PTKs and PTPs in the regulation of mitogenesis,

neoplastic transformation, cellular differentiation and ion channel regulation is well established (Hunter and Cooper, 1985; Cantley *et al.*, 1991; Holmes *et al.*, 1996; Neel and Tonks, 1997), little is known about how they may act in concert to produce a defined cellular response.

In recent years, our laboratory and others have provided some new insights into the role of PTKs in the control of ion channel activity. The m1 muscarinic acetylcholine receptor, a G protein-coupled receptor (GPCR), previously was shown to stimulate a cellular tyrosine kinase activity that leads to the direct tyrosine phosphorylation and suppression of a voltage-gated potassium channel, Kv1.2 (Huang *et al.*, 1993). m1 receptor signaling through either the epidermal growth factor (EGF) receptor (Tsai *et al.*, 1997) or the PYK2 tyrosine kinase (Lev *et al.*, 1995; Felsch *et al.*, 1998) subsequently was shown to participate in channel suppression. Additionally, the Src tyrosine kinase was found to bind to, phosphorylate and suppress the activity of the related Kv1.5 potassium channel (Holmes *et al.*, 1996). Tyrosine phosphorylation influences the activity of other ion channels, including the inositol trisphosphate (IP₃)-gated Ca²⁺ channel (Jayaraman *et al.*, 1996), and the ligand-gated nicotinic acetylcholine (Hopfield *et al.*, 1988) and NMDA (*N*-methyl-D-aspartate) channels (Wang and Salter, 1994). While these studies demonstrate a pivotal role for PTKs in ion channel regulation, the function of PTPs in similar instances remains largely uncharacterized. In fact, although PTP activities have been shown to control ion channel activity (Wilson and Kaczmarek, 1993; Wang and Salter, 1994; Catarsi and Drapeau, 1997), the molecular identities of the phosphatases remain unknown. Here we report that a previously cloned transmembrane tyrosine phosphatase, receptor protein tyrosine phosphatase α (RPTP α), participates in the m1 mAChR-mediated regulation of Kv1.2 activity.

RPTP α belongs to the family of receptor protein tyrosine phosphatases (RPTPs) that contain a single membrane-spanning region with two cytoplasmic catalytic PTP domains (reviewed in Neel and Tonks, 1997). It is thought that the proximal PTP domain exerts the predominant catalytic activity while the distal PTP domain directs protein-protein interactions (Pulido *et al.*, 1995; Serra-Pages *et al.*, 1995). In addition, the cytoplasmic domain of RPTP α is subjected to both serine phosphorylation (den Hertog *et al.*, 1995; Tracy *et al.*, 1995) and tyrosine phosphorylation (den Hertog *et al.*, 1994; Su *et al.*, 1994). The extracellular domain of RPTP α is heavily glycosylated but, unlike RPTPs involved in cell-cell or cell-matrix adhesion events, RPTP α lacks the immunoglobulin-like or type III fibronectin domains that mediate these events. Interestingly, although RPTP α is found in many tissues and is particularly abundant in the central nervous system (Sap *et al.*, 1990), a functional ligand has not been

Fig. 1. Vanadate pre-treatment enhances the m1 mAChR-dependent suppression of Kv1.2-generated K^+ current in m1K-293 cells. For each condition, recordings from five cells were averaged and normalized to the steady-state current measured at 100 ms generated by a step to +60 mV in cells treated with neither vanadate or carbachol (A, left). Steady-state I-V curves are depicted to the right of the current traces [■ = (-) carbachol; ● = (+) carbachol]. (A) In cells not pre-treated with vanadate, application of 1 mM carbachol produces a 38% suppression of Kv1.2 current generated by a family of voltage steps from -70 to +60 mV. (B) In contrast, 1 mM carbachol elicits an 85% suppression in cells that had been pre-treated with 1 mM vanadate for at least 10 min.

identified. Therefore, it is possible that intracellular signaling pathways, similar to those described for transactivation of the EGF receptor (Daub *et al.*, 1996; Tsai *et al.*, 1997), may constitute one mechanism by which RPTP α is regulated.

Previously identified substrates for RPTP α include c-src and the insulin receptor (Zheng *et al.*, 1992; Moller *et al.*, 1995). Our findings expand that to include the Shaker family potassium channel, Kv1.2. Through studies in transfected human 293 cells stably expressing the m1 receptor and the potassium channel Kv1.2, we found that m1 mAChR stimulation increases the tyrosine phosphorylation of endogenous RPTP α through a protein kinase C (PKC)-dependent pathway. Co-immunoprecipitation and *in vitro* binding experiments revealed that m1 mAChR stimulation also triggers the physical association of RPTP α with the Kv1.2 channel. Biochemical experiments in 293 cells demonstrate that receptor-induced association of RPTP α with Kv1.2 results in a reversal of tyrosine kinase-dependent phosphorylation of Kv1.2, and electrophysiological analysis reveals an ensuing reversal of tyrosine kinase-dependent channel suppression. Furthermore, RPTP α was shown to increase the basal current of Kv1.2 in *Xenopus* oocytes. Taken together, these findings identify RPTP α as a new target of m1 mAChR signaling and reveal a novel regulatory mechanism involving a transmembrane PTP in GPCR-dependent regulation of a potassium channel.

Results

Tyrosine phosphatases regulate the activity of Kv1.2

In transfected 293 cells stably expressing the m1 mAChR, the Kv1.2 α subunit and the Kv β 2 subunit (hereafter

referred to as m1K-293 cells), stimulation of the m1 receptor with the acetylcholine analog, carbachol, activates a cellular tyrosine kinase activity which leads to the tyrosine phosphorylation and suppression of the Kv1.2 potassium channel (Huang *et al.*, 1993; Tsai *et al.*, 1997; Cachero *et al.*, 1998). However, Kv1.2 channels undergo a mean carbachol-dependent suppression of 68% when expressed in *Xenopus* oocytes (Huang *et al.*, 1993), but only 38% suppression when expressed in 293 cells (1.3 ± 0.1 nA at 0 mV in cells not treated with carbachol versus 0.8 ± 0.1 nA in cells treated with 1 mM carbachol, $n = 5$) (Figure 1A; Tsai *et al.*, 1997; Cachero *et al.*, 1998). As direct tyrosine phosphorylation of the channel is thought to be required for its suppression, we hypothesized that the relatively weak m1 receptor-mediated suppression of Kv1.2 activity in 293 cells might be due to a competing tyrosine phosphatase activity acting on the channel. To test this possibility, we pre-treated cells with vanadate, a non-specific tyrosine phosphatase inhibitor. Such treatment results in a dramatic enhancement of m1 mAChR-induced suppression of Kv1.2 current (1.3 ± 0.3 nA at 0 mV in cells not treated with carbachol versus 0.2 ± 0.1 nA in cells treated with 1 mM carbachol, $n = 5$) (Figure 1B). This experiment suggests that, in addition to PTKs, PTPs may also be involved in the regulation of Kv1.2 activity.

Tyrosine phosphorylation and association of RPTP α with Kv1.2

To test further the idea that PTPs regulate Kv1.2 activity, we asked if m1 receptor-mediated tyrosine phosphorylation of Kv1.2 is enhanced similarly in the presence of vanadate. Kv1.2 protein immunoprecipitated from m1K-293 cells

pre-treated with or without vanadate and subsequently treated with or without carbachol were subjected to Western blot analysis with an anti-phosphotyrosine-specific monoclonal antibody (4G10). The results revealed that carbachol-induced tyrosine phosphorylation of the 63 kDa Kv1.2 protein was enhanced in the presence of vanadate (Figure 2A, top; Huang *et al.*, 1993), consistent with the hypothesis that PTPs regulate the phosphotyrosine content and, thereby, the activity of Kv1.2. Interestingly, carbachol treatment also stimulated the tyrosine phosphorylation of two additional proteins of ~80 and 130 kDa which co-precipitated with Kv1.2 (Figure 2A, top).

RPTP α , reported to be either a 100 or a 130 kDa protein due to differential glycosylation (Daum *et al.*, 1994), can act as a substrate for the c-src tyrosine kinase

(den Hertog *et al.*, 1994). Since the m1 receptor signaling pathway can activate c-src in m1K-293 cells (Tsai *et al.*, 1997), we hypothesized that the 130 kDa protein might be RPTP α . To test this possibility directly, we stripped and re-probed the same nitrocellulose filter with an RPTP α -specific monoclonal antibody and found that a 130 kDa band became labeled (Figure 2A, middle). Importantly, the extent of RPTP α binding to Kv1.2 appeared to increase in response to carbachol stimulation. In order to test the specificity of this interaction, we asked whether Kv1.2 binds to another receptor tyrosine phosphatase, RPTP β , or a cytoplasmic tyrosine phosphatase, SHP-2, which is known to be a downstream target of G protein-coupled receptor signaling (Ali *et al.*, 1997). Immunoprecipitates of Kv1.2 from m1K-293 cells treated with or without carbachol revealed no interaction between Kv1.2 and either RPTP β or SHP-2 (Figure 2B). Thus, activation of the m1 receptor increased both the tyrosine phosphorylation of RPTP α and Kv1.2 as well as their specific association. The molecular identity of the p80 protein remains unknown.

To explore the specificity of the m1 receptor-regulated association of RPTP α and the Kv1.2 protein, we tested whether the endogenous EGF and insulin receptors can also induce this association. Since both of these receptors have been shown to induce cellular tyrosine kinase activities (reviewed in Ullrich and Schlessinger, 1990), they potentially may regulate the RPTP α -Kv1.2 interaction. Co-immunoprecipitation experiments showed that, while the carbachol-induced phosphatase-channel interaction was blocked by the muscarinic-specific antagonist, atropine, neither EGF nor insulin induced such association (Figure 2C). Therefore, the effect of m1 receptor signaling on RPTP α and Kv1.2 exhibits a degree of specificity among other transmembrane receptors.

Finally, to establish the physiological relevance of the Kv1.2-RPTP α interaction observed in a cell culture system, we performed similar co-immunoprecipitation experiments using whole-cell lysates derived from rat brain. Western blot analysis revealed that endogenous

Fig. 2. The m1 mAChR induces the tyrosine phosphorylation and association of RPTP α with Kv1.2 in m1K-293 cells. **(A)** m1K-293 cells pre-treated with or without 1 mM vanadate for 10 min were stimulated subsequently without (control) or with carbachol (1 mM, 20 min). Cells immediately were lysed, and ~1 mg of protein was incubated with 1 μ g of anti-Kv1.2 antibody. The immunoprecipitates were resolved by 7.5% SDS-PAGE, transferred to nitrocellulose and immunoblotted with either an anti-phosphotyrosine antibody (top), an anti-RPTP α antibody (middle) or anti-Kv1.2 antibody (bottom). Arrows indicate the positions of p130 RPTP α , p80 (unidentified protein) and p63 Kv1.2. **(B)** Unstimulated m1K-293 cells (control) and cells stimulated with 1 mM carbachol for 20 min (carb) were lysed. Anti-Kv1.2 immunoprecipitates were analyzed as above with the appropriate antibody. Arrows indicate the positions of p250 RPTP β , p70 SHP-2 and p63 Kv1.2. **(C)** Unstimulated m1K-293 cells (control), cells stimulated with 1 mM carbachol for 20 min (carb), cells treated with 100 μ M atropine (a mAChR antagonist) and 1 mM carbachol for 20 min (atr/carb), cells treated with 10 ng/ml EGF for 20 min (EGF) and cells treated with 1 μ M insulin for 20 min (Ins) were lysed. Anti-Kv1.2 immunoprecipitates were analyzed as above with the appropriate antibody. Arrows indicate the positions of p130 RPTP α and p63 Kv1.2. **(D)** A 500 μ g aliquot of cell lysates derived from rat brain was incubated with or without 1 μ g of anti-Kv1.2 antibody. Anti-Kv1.2 immunoprecipitates were analyzed as above. Arrows indicate the positions of p100 RPTP α (a differentially glycosylated form) and p63 Kv1.2. Similar results were obtained in two additional experiments.

Fig. 3. RPTPα tyrosine phosphorylation in response to m1 mAChR stimulation requires PKC-dependent signals. (A) Unstimulated m1K-293 cells (control), cells stimulated with 1 mM carbachol for 20 min (carb), cells treated with 100 μM atropine and 1 mM carbachol for 20 min (atr/carb), cells treated with 10 ng/ml EGF for 20 min (EGF) and cells treated with 1 μM insulin for 20 min (Ins) were lysed. Anti-RPTPα immunoprecipitates were analyzed with the appropriate antibody. Arrows indicate the position of p130 RPTPα. (B) Unstimulated m1K-293 cells (control), cells stimulated with 1 mM carbachol for 20 min (carb), cells stimulated with 10 μM PMA for 20 min (PMA), cells stimulated with 10 μM A23187 for 20 min (A23187), cells pre-treated with 15 μM GF109203X (a protein kinase C inhibitor) for 30 min and challenged with 1 mM carbachol for 20 min (GF109203X/carb), and cells pre-treated with 0.5 mM EDTA for 20 min and challenged with 1 mM carbachol for 20 min (EDTA/carb) were lysed. Anti-RPTPα immunoprecipitates were analyzed as above with the appropriate antibody. Arrows indicate the position of p130 RPTPα. Similar results were obtained in two additional experiments.

Kv1.2 associates with the endogenous 100 kDa form of RPTPα (Figure 2D). Therefore, the channel–phosphatase interaction occurs in native tissues.

RPTPα tyrosine phosphorylation requires PKC-dependent signals

The studies presented in Figure 2 predict that directly immunoprecipitated RPTPα should show an increase in its phosphotyrosine content following carbachol treatment. Western blot analysis of RPTPα immunoprecipitates with a phosphotyrosine-specific antibody confirmed this prediction as carbachol treatment significantly induced the tyrosine phosphorylation of the p130 kDa band corresponding to RPTPα (Figure 3A). The identity of the lower band which also becomes tyrosine phosphorylated in response to carbachol and, to a lesser extent, EGF treatment remains uncharacterized (Figure 3A).

The m1 mAChR couples to the $G_{\alpha q}$ subtype of heterotrimeric G proteins to stimulate phospholipase C (PLC) activity which in turn leads to both the activation of PKC and the release of Ca^{2+} from intracellular stores (Lechleiter *et al.*, 1990a,b; Bernstein *et al.*, 1992). To test the role of PKC in the m1 mAChR-mediated RPTPα tyrosine phosphorylation, we treated m1K-293 cells with the PKC activator, phorbol 12-myristate 13-acetate (PMA). Such treatment increased the phosphotyrosine content of immunoprecipitated RPTPα, indicating that activation of PKC is sufficient to cause tyrosine phosphorylation of RPTPα (Figure 3B). In cells pre-treated for 20 min with the specific PKC inhibitor, GF109203X (Toullec *et al.*, 1991), carbachol-induced tyrosine phosphorylation of RPTPα was significantly reduced (Figure 3B). Therefore, PKC activation appears to be both necessary and sufficient for m1 mAChR-induced tyrosine phosphorylation of RPTPα.

Since carbachol induces large increases in cytoplasmic Ca^{2+} in m1K-293 cells stably expressing the m1 receptors (Cachero *et al.*, 1998), we asked whether elevated Ca^{2+} plays an important role in coupling m1 receptor activation

to RPTPα tyrosine phosphorylation. The Ca^{2+} ionophore, A23187, failed to induce tyrosine phosphorylation of RPTPα (Figure 3B), even though direct Ca^{2+} imaging analysis confirmed that ionophore treatment resulted in a rapid increase in Ca^{2+} (Cachero *et al.*, 1998). Therefore, Ca^{2+} influx is not sufficient to cause RPTPα tyrosine phosphorylation. Interestingly, carbachol failed to elicit an increase in the phosphotyrosine content of RPTPα in cells with media containing the Ca^{2+} chelator, EDTA (Figure 3B). Since application of extracellular EDTA can lower the cytoplasmic resting Ca^{2+} concentration, intracellular Ca^{2+} may play an indirect role in mediating RPTPα tyrosine phosphorylation. Nevertheless, our results suggest that m1 receptor-induced tyrosine phosphorylation of RPTPα relies primarily on PKC-dependent signals.

Both the N- and C-termini of Kv1.2 mediate RPTPα binding

The Kv1.2 protein is a single polypeptide of 499 amino acids (Paulmichl *et al.*, 1991). To investigate whether the cytosolic N- or C-termini of Kv1.2 associate with RPTPα, we analyzed the ability of GST fusion proteins containing either the N-terminal domain (NTD: amino acids 1–163) or C-terminal domain (CTD: amino acids 412–499) of Kv1.2 to bind to RPTPα. Each GST fusion protein was purified from bacteria by immobilization on glutathione-conjugated agarose beads and incubated with total cell extracts derived from m1K-293 cells treated with or without carbachol. Non-carbachol-treated and carbachol-treated cell lysates, in principle, should contain non-tyrosine-phosphorylated and tyrosine-phosphorylated RPTPα proteins, respectively (Figure 3). Following washing, proteins bound to the agarose beads were subjected to Western blot analysis using an RPTPα-specific antibody. We found that the NTD associates with RPTPα only from control cell lysates and not from carbachol-treated lysates (Figure 4A, top, lanes 4 and 5), while the CTD binds to RPTPα from both control and, to a greater extent, carbachol-treated lysates (Figure 4A, top, lanes 6 and 7).

Fig. 4. RPTP α binding to Kv1.2 N- and C-termini *in vitro*. (A) GST fusion proteins purified from BL21 bacterial cells immobilized on glutathione-conjugated agarose beads were incubated with cell lysates derived from m1K-293 cells treated without carbachol (control) or with 1 mM carbachol for 20 min (carb). After washing, proteins bound to beads were released by boiling and separated by SDS-PAGE. RPTP α and RPTP β proteins were detected by immunoblotting with anti-RPTP α (top) or anti-RPTP β antibody (middle), and GST fusion proteins were detected by Coomassie staining (bottom). Arrows point to the position of p130 RPTP α and p250 RPTP β . GST proteins produced in BL21 cells are not tyrosine phosphorylated, as confirmed below. (B) GST proteins purified from BL21 and TKB1 bacterial cells are supposed to produce non-tyrosine-phosphorylated and tyrosine-phosphorylated proteins, respectively. This was confirmed by subjecting the fusion proteins to SDS-PAGE followed by immunoblotting with an anti-phosphotyrosine antibody. (C) GST fusion proteins purified from TKB1 bacterial cells immobilized on glutathione-conjugated agarose beads were incubated with cell lysates derived from m1K-293 cells treated without carbachol (control) or with 1 mM carbachol for 20 min (carb). After washing, proteins bound to beads were released by boiling and separated by SDS-PAGE. RPTP α protein was detected by immunoblotting with anti-RPTP α antibody (top) and the GST fusion proteins were detected by Coomassie staining (bottom). The arrow points to the position of p130 RPTP α . Similar results were obtained in two additional experiments.

GST alone did not bind to RPTP α from either the control or carbachol-treated lysates (Figure 4A, top, lanes 2 and 3). We re-probed the nitrocellulose filter with an RPTP β -specific monoclonal antibody and detected no signal (Figure 4A, middle). A Coomassie gel shows that similar amounts of GST fusion proteins were used during the experiment (Figure 4A, bottom). We conclude from these studies that Kv1.2's NTD binds to non-tyrosine-phosphorylated RPTP α , while the CTD associates with both non-tyrosine-phosphorylated and, with slightly higher affinity, tyrosine-phosphorylated RPTP α .

As tyrosine phosphorylation of RPTP α appears to affect binding to Kv1.2's NTD and CTD, we asked whether the tyrosine phosphorylation state of Kv1.2 also plays a role in this interaction. In principle, m1 receptor-stimulated tyrosine phosphorylation of Kv1.2 can take place on any combination of tyrosine residues located on the NTD

(tyrosines 23, 68, 76, 90, 91, 92, 116 and 132) and/or CTD (tyrosines 415, 417, 429, 458 and 489) of Kv1.2. To address the importance of tyrosine phosphorylation of these residues in mediating RPTP α binding, we purified tyrosine-phosphorylated GST fusion proteins from a bacterial cell line harboring an inducible plasmid encoding the elk tyrosine kinase (Lhotak *et al.*, 1991). We tested the viability of this strain by purifying GST fusion proteins from either the wild-type (BL21) or elk tyrosine kinase (TK)-expressing (TKB1) bacterial strains and confirmed that only the NTD and CTD of the GST fusion proteins purified from the TK-expressing bacterial cells produced phosphotyrosine proteins (Figure 4B). Surprisingly, while unphosphorylated NTD binds RPTP α only in unstimulated cells (Figure 4A, top, lanes 4 and 5), tyrosine-phosphorylated NTD binds to RPTP α only from stimulated cells (Figure 4C, top, lanes 4 and 5). In contrast, unphosphoryl-

ated CTD and tyrosine-phosphorylated CTD displayed a similar RPTP α -binding pattern (compare Figure 4A, top, lanes 6 and 7, and C, top, lanes 6 and 7). GST purified from TKB1 cells did not bind to RPTP α from either the control or carbachol-treated lysates (Figure 4C, top, lanes 2 and 3). A Coomassie gel shows similar amounts of fusion proteins used during the experiment (Figure 4C, bottom). Since tyrosine phosphorylation of Kv1.2's NTD increased its binding to RPTP α derived from carbachol-

treated lysates, we conclude that phosphorylation of tyrosine residues on the N-terminus of Kv1.2 as well as RPTP α appears to be critical in stabilizing the channel-phosphatase interaction; this interaction is disrupted when only one protein is tyrosine phosphorylated (Figure 4A, lane 5, and C, lane 4). However, since the RPTP α -binding pattern using non-tyrosine-phosphorylated and tyrosine-phosphorylated CTD of Kv1.2 was similar, tyrosine phosphorylation of Kv1.2's C-terminus does not appear to be necessary for the interaction. Instead, tyrosine phosphorylation of RPTP α may play a role in the enhanced association. Nevertheless, these studies demonstrate that both termini of Kv1.2 can associate specifically with RPTP α *in vitro*.

Functional interaction of RPTP α with Kv1.2

Although co-immunoprecipitation and *in vitro* binding experiments demonstrate a specific physical interaction between RPTP α and the Kv1.2 protein, it remained unclear whether RPTP α can dephosphorylate and thereby increase the amplitude of current generated by the Kv1.2 channel. To test directly the possibility that m1 receptor-induced association of RPTP α with Kv1.2 can potentiate Kv1.2 activity, we took advantage of PMA's ability to potentially suppress Kv1.2 current via a tyrosine kinase-dependent pathway without recruiting RPTP α to the channel (Huang *et al.*, 1993; Figure 5A and B). The degree of PMA-induced phosphorylation and suppression of Kv1.2 activity in the absence of vanadate (Figure 5A and B, lanes 2 and 5) was similar to carbachol-mediated Kv1.2 phosphorylation and suppression in the presence of vanadate (Figures 1B and 2A). Since the latter reflects the full extent of m1 receptor-induced tyrosine kinase activity acting on the channel, we reasoned that PMA stimulation mimicks the tyrosine kinase, but not the tyrosine phosphatase, component of the m1 receptor signaling pathway. This idea is supported by the finding that PMA did not induce Kv1.2-RPTP α association (Figure 5B, lanes 2 and 5). Therefore, we asked whether carbachol-mediated association of RPTP α with PMA-suppressed Kv1.2 channels may reverse PMA-induced phosphorylation and suppression of Kv1.2. In m1K-293 cells transiently transfected with a plasmid encoding RPTP α (mean steady-state current at 0 mV for untreated cells = 984 ± 107 pA, $n = 9$), carbachol significantly reversed the PMA-induced current

Fig. 5. Functional interaction of RPTP α and Kv1.2. **(A)** Graphs represent the mean steady-state current elicited by a pulse to 0 mV and normalized to values obtained in control cells. Error bars represent standard error of the mean. Carbachol fails to reverse PMA-induced suppression in cells transfected with GFP (left). In contrast, carbachol produced a significant increase in PMA-suppressed current in cells that had been transfected with RPTP α (right). **(B)** m1K-293 cells were transiently transfected with either a control (GFP) or RPTP α plasmid. Lysates from unstimulated cells (control), cells treated with 1 nM PMA (PMA) or cells treated with 1 nM PMA for 10 min followed by 1 mM carbachol for 20 min (PMA/carb) and immunoprecipitated with 1 μ g of anti-Kv1.2 antibody were subjected to Western blot analysis with either an anti-phosphotyrosine antibody (top), an anti-RPTP α antibody (middle) or an anti-Kv1.2 antibody (bottom). Arrows indicate the position of p130 RPTP α , p63 Kv1.2 and p80 (unknown). Similar results were obtained in two additional experiments. **(C)** Currents were evoked with a pulse from -80 mV to +30 mV in 10 mV increments from a holding potential of -60 mV. Histograms depict the steady-state current measured at 0 mV from oocytes injected with RNA for Kv1.2 and either RPTP α or H₂O ($n = 12$ for each condition).

suppression in cells sequentially treated with PMA (1 nM, 10 min) and then carbachol (1 mM, 20 min) (234 ± 45 versus 479 ± 58 pA, *t*-test $P < 0.001$, $n = 9$) (Figure 5A, right). In cells transiently transfected with a control plasmid (green fluorescence protein: GFP) (mean steady-state current at 0 mV for untreated cells = 1385 ± 185 pA, $n = 9$), carbachol had no significant effect on PMA-suppressed current (413 ± 92 versus 270 ± 47 pA, *t*-test $P > 0.08$, $n = 9$) (Figure 5A, left; see Discussion). In cells overexpressing RPTP α , carbachol induced the tyrosine phosphorylation of RPTP α and its association with Kv1.2 in cells pre-treated with PMA, which corresponded to a dramatic decrease in Kv1.2's phosphotyrosine content (Figure 5B, compare lanes 5 and 6). However, in cells overexpressing GFP, carbachol failed significantly to induce the tyrosine phosphorylation of RPTP α and its recruitment to Kv1.2 when pre-treated with PMA, which corresponded to a retention of Kv1.2's high phosphotyrosine content (Figure 5B, compare lanes 2 and 3). The inverse correlation between Kv1.2's phosphotyrosine content and the presence of RPTP α suggests that the channel probably serves as an *in vivo* substrate for the phosphatase. Therefore, carbachol-dependent recruitment of RPTP α to Kv1.2 dephosphorylates Kv1.2 channels, thereby reversing the suppression of Kv1.2 activity.

To demonstrate further that RPTP α can affect Kv1.2 physiology, we performed electrophysiology in a different model system, namely *Xenopus* oocytes. Oocytes were co-injected with cRNAs encoding Kv1.2 alone or with RPTP α . Oocytes co-expressing Kv1.2 and RPTP α generated currents that were larger than currents generated in oocytes expressing Kv1.2 alone ($n = 12$, $P < 0.01$, one-tailed *t*-test) (Figure 5C). The average of the mean steady-state amplitude of Kv1.2 current evoked by a pulse to +30 mV was 18 ± 2 μ A in control oocytes and 25 ± 2 μ A in those co-injected with RPTP α . The percentage increase in whole-cell currents was 36% for RPTP α co-injection. These data indicate that RPTP α can stimulate Kv1.2 activity and, furthermore, are consistent with our findings in 293 cells demonstrating a functional interaction between the channel and the phosphatase.

Discussion

The studies described here suggest that RPTP α plays a role in neurotransmitter signaling. In particular, they indicate a novel role for RPTP α in regulating the m1 mAChR-dependent modulation of an ion channel activity. We previously had shown that carbachol stimulation of 293 cells stably expressing both the m1 receptor and the Kv1.2 channel induced the tyrosine phosphorylation of the Kv1.2 protein, thereby leading to its suppression (Huang *et al.*, 1993). More recently, we have found that carbachol stimulation of m1 receptors in 293 cells produces only partial current suppression when compared with Kv1.2 suppression in *Xenopus* oocytes (Huang *et al.*, 1993). We reasoned that one explanation for this difference may be the existence of a steady-state or receptor-activated tyrosine phosphatase activity in 293 cells that acts on Kv1.2. Consistent with this idea, we found that a transmembrane tyrosine phosphatase RPTP α becomes tyrosine phosphorylated, and co-precipitates with and dephosphorylates Kv1.2 in an m1 receptor-regulated manner.

Therefore, in 293 cells, m1 receptor stimulation triggers not only a tyrosine kinase activity that phosphorylates Kv1.2, but also a tyrosine phosphatase activity that can act in opposition to it.

The m1 receptor potently activates PLC β , and we therefore tested the importance of PKC and intracellular Ca^{2+} in m1 receptor-mediated tyrosine phosphorylation of RPTP α . Activation of PKC by the phorbol ester PMA caused a strong increase in the phosphotyrosine level of RPTP α , while application of a PKC inhibitor, GF109203X, dramatically blocked RPTP α tyrosine phosphorylation by carbachol, indicating that PKC is both necessary and sufficient for m1 receptor-mediated phosphorylation of RPTP α . In contrast, application of the Ca^{2+} ionophore, A23187, failed to induce tyrosine phosphorylation of RPTP α . Thus, influx of intracellular Ca^{2+} does not appear to generate a sufficient signal to induce this process. External application of the Ca^{2+} chelator, EDTA, blocked carbachol-mediated RPTP α tyrosine phosphorylation. This finding is also consistent with a role for PKC in this process since extracellular treatment with EDTA can lower the resting cytoplasmic Ca^{2+} concentration to levels that disrupt normal receptor-dependent PKC activation (reviewed in Newton, 1995).

We next investigated whether the m1 receptor-induced tyrosine phosphorylation of RPTP α could affect its catalytic activity. Direct immunoprecipitation of RPTP α revealed that its phosphotyrosine level increased significantly in response to carbachol stimulation. Based on an *in vitro* PTP assay using a synthetic phosphotyrosine peptide as a substrate, we found that tyrosine phosphorylation of RPTP α did not alter its catalytic activity significantly (not shown). This finding is consistent with previous studies showing that serine but not tyrosine phosphorylation stimulated RPTP α 's catalytic activity (den Hertog *et al.*, 1994, 1995). Although tyrosine phosphorylation of PTPs can induce their catalytic activation in some instances (Vogel *et al.*, 1993; Stover and Walsh, 1994), this effect can be difficult to measure since PTPs undergo autodephosphorylation (Stein-Gerlach *et al.*, 1995). Interestingly, the crystal structure of the murine RPTP α 's proximal catalytic domain suggests a dimerization-induced mechanism for its catalytic inhibition (Bilwes *et al.*, 1996). Whether RPTP α 's monomer-dimer ratio is altered in response to m1 receptor signaling remains to be determined.

As tyrosine phosphorylation of RPTP α by the m1 receptor does not appear to change its catalytic properties, such phosphorylation may instead play a role in targeting RPTP α to downstream effector proteins. In fact, phosphorylation of Tyr789 on RPTP α is necessary for recruiting the Grb2 adaptor protein via an SH₂ domain-dependent interaction (den Hertog *et al.*, 1994). Although neither Kv1.2 nor RPTP α contain SH₂ or PTB domains, structures that normally mediate phosphotyrosine-specific protein-protein interactions (Pawson, 1995), tyrosine phosphorylation of both proteins does appear to play a role in stabilizing their interaction. Co-immunoprecipitation studies revealed that m1 receptor-induced association of RPTP α and Kv1.2 was coincident with an increase in the phosphotyrosine content of both proteins. The *in vitro* binding experiments depicted in Figure 4 demonstrate that RPTP α and the C-terminus of Kv1.2 associated with each other regardless of their relative tyrosine phosphorylation state, but RPTP α

and the N-terminus of Kv1.2 interacted only when both proteins were in the same state of tyrosine phosphorylation. We currently are generating point mutants within Kv1.2's N-terminus in order to identify the specific tyrosine site(s) that mediate this interaction. While it is tempting to suggest that tyrosine phosphorylation of Kv1.2's N-terminus acts as a switch to trigger RPTP α association, we do not know whether this behavior occurs in the presence of Kv1.2's C-terminus. Whether the N- and C-termini of Kv1.2 coordinately mediate Kv1.2 interaction remains to be characterized. Nevertheless, tyrosine phosphorylation-dependent association of RPTP α with the N-terminus of Kv1.2 is of particular interest given the importance of Tyr132 within Kv1.2's N-terminus during receptor-mediated channel suppression (Huang *et al.*, 1993). Since phosphorylation of a specific tyrosine residue in Kv1.2's N-terminal domain (i.e. Tyr132) regulates its activity, the ability of a tyrosine phosphatase to bind to this same domain should, in principle, permit efficient dephosphorylation of the same site. Therefore, while tyrosine phosphorylation has no apparent effect on RPTP α 's catalytic activity, it appears to play an important role by recruiting the existing catalytic activity of RPTP α into close proximity with its substrate, Kv1.2.

Although tyrosine phosphorylation is important for the phosphatase-channel association, it is unlikely to be the only factor involved. Both m1 receptor stimulation and activation of PKC with PMA trigger the tyrosine phosphorylation of RPTP α as well as the tyrosine phosphorylation and suppression of Kv1.2 (Huang *et al.*, 1993; this study). However, unlike m1 receptor activation, PMA does not induce the co-precipitation of these two proteins. It appears, therefore, that tyrosine phosphorylation is not sufficient to promote the phosphatase-channel association. Additional factors necessary for this interaction may involve other cellular proteins regulated by the m1 receptor signaling pathway. For example, the physical association of the small GTP-binding protein RhoA with the N-terminus of Kv1.2 recently was shown to mediate m1 receptor-dependent suppression of Kv1.2 (Cachero *et al.*, 1998). Such an interaction may add an additional level of control over Kv1.2-RPTP α association within the cell.

Finally, to examine the functional significance of Kv1.2-RPTP α interaction, we took two different approaches. First, we used a *Xenopus* oocyte expression system to test the effect of RPTP α on Kv1.2 current. We found that co-expression of RPTP α increases basal Kv1.2 current, suggesting a physiological role for RPTP α on Kv1.2. Secondly, using 293 cells, we took advantage of the ability of PMA potently to suppress Kv1.2 current while not inducing RPTP α -Kv1.2 association. Since a previous study had shown that PMA induces tyrosine kinase-dependent suppression of Kv1.2 (Huang *et al.*, 1993), we asked whether m1 receptor-triggered co-association of RPTP α with Kv1.2 could reverse PMA-induced suppression of Kv1.2. This idea assumes that any further m1 receptor-stimulated tyrosine kinase activity will be negligible due to prior activation by PMA. Such an assumption is supported by the observation that carbachol-dependent phosphorylation and suppression of Kv1.2 in the presence of vanadate, which essentially unmasks the full extent of m1 receptor-induced tyrosine kinase activity acting on the channel, is similar to the PMA-induced effect in 293 cells

(compare Figures 1B and 2A with Figure 5A and B). Electrophysiological analysis demonstrated that, in cells overexpressing RPTP α but not a control plasmid (GFP), addition of carbachol following PMA pre-treatment significantly reversed the suppression of Kv1.2 current (Figure 5A). The molecular basis for such a reversal can be observed biochemically as, under the same conditions, RPTP α strongly interacted with Kv1.2 channels whose phosphotyrosine content were dramatically decreased (Figure 5B). The correlation between the reversal of the channel's activity, the decrease in its phosphotyrosine content and the presence of RPTP α suggests a scenario in which recruitment of RPTP α leads to the dephosphorylation of Kv1.2, resulting in the stimulation of its current activity.

In cells pre-treated with PMA, carbachol-induced reversal of Kv1.2 current can only be achieved when sufficient RPTP α is recruited to the channel to dephosphorylate it strongly. Endogenous RPTP α did not provide sufficient phosphatase activity either to dephosphorylate Kv1.2 or to reverse channel suppression. Instead, overexpression of RPTP α was necessary to achieve these effects. In one scenario, the absence of strong dephosphorylation via endogenous RPTP α may result from an insufficient amount of native RPTP α protein available to recruit to overexpressed Kv1.2. Alternatively, PMA pre-treatment may recruit signaling molecules to the channel which sterically hinder the subsequent recruitment of endogenous RPTP α ; overexpressing RPTP α might overcome such steric hindrance.

At present, we do not know whether RPTP α is the only tyrosine phosphatase regulating Kv1.2 activity. It is formally possible that channel dephosphorylation is caused not by RPTP α *per se*, but by another phosphatase that may be recruited via its association with RPTP α . As is the case for other PTPs (Bennett *et al.*, 1994; Li *et al.*, 1994), RPTP α may serve as an adaptor molecule by linking other regulatory components to Kv1.2, and these molecules may be equally important for the effects that we report.

Taken together, these findings demonstrate the ability of Kv1.2 to serve as an *in vivo* substrate for RPTP α and show that modulation of Kv1.2 channel activity ultimately relies on the dynamic balance between receptor-stimulated tyrosine kinase and tyrosine phosphatase activity.

Materials and methods

Reagents and plasmids

Antibodies were purchased from the following vendors: Upstate Biotechnology (4G10-HRP), Transduction Laboratories (anti-RPTP α and anti-RPTP β) and Santa Cruz Biotechnology (anti-SHPTP-2). Anti-Kv1.2 antibody was generated in our laboratory. Protein G- and protein A-Sepharose beads were purchased from Sigma and Pharmacia, respectively. The PKC activator, PMA, and the Ca²⁺ ionophore, A23187, were purchased from Sigma. The PKC inhibitor, GF109203X, was purchased from Research Biochemical International. The plasmid encoding the RPTP α gene was a generous gift of Dr J.Sap (New York University).

Cell culture

Human embryonic kidney 293 cells were transfected by the calcium phosphate method. Stable cell lines expressing the m1 mAChR, Kv1.2 α subunit and Kv β 2 subunit were obtained as described in Peralta *et al.* (1988). All of the experiments in this study were performed in this cell line, referred in the text as m1K-293 cells. RPTP α , RPTP β , SHP-2, EGF receptors and insulin receptors are endogenously expressed by these cells. The 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS),

100 U/ml penicillin and streptomycin, and 2 mM L-glutamine, and selected with 500 μ g/ml of G418 and 200 μ g/ml of Zeocin.

Immunoprecipitation and immunoblot analysis

m1K-293 cells were grown to confluency in 60 mm dishes. Cells were treated subsequently with the appropriate ligand and/or drug for varying lengths of time as described in the figure legends. Cells were lysed in a modified RIPA buffer containing Tris-HCl (50 mM, pH 7.4), 1% NP-40, sodium deoxycholate (0.25%), sodium chloride (150 mM), EGTA (1 mM), phenylmethylsulfonyl fluoride (PMSF; 1 mM), leupeptin (10 μ g/ml), aprotinin (10 μ g/ml), vanadate (1 mM) and sodium fluoride (1 mM). Rat brains (adult male Lewis rats) were lysed in the same buffer. After incubating for 30 min at 4°C, the cell lysates were centrifuged at 12 000 g for 10 min and the supernatant was collected. For immunoprecipitation experiments, 1–2 μ g of the appropriate antibody were added to the samples and incubated for 6 h at 4°C followed by the addition of either protein G-agarose or protein A-Sepharose beads for 10 h at 4°C. Antibody-antigen complexes were washed twice with the RIPA buffer and once with a buffer containing Tris-HCl (50 mM, pH 7.4) and EDTA (1 mM). The samples were eluted by boiling in SDS sample buffer. The immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with the desired antibody. When appropriate, the nitrocellulose membranes were stripped and reprobed with another antibody.

In RPTP α transient expression studies (Figure 5), ~5 μ g of a plasmid encoding the control lacZ or RPTP α plasmid was transfected into m1K-293 cells by using the calcium phosphate method. After growing the cells in an incubator containing 0% CO₂ for 6 h, fresh DMEM was replenished and the cells grew for an additional 48 h in an incubator containing 5% CO₂. The Kv1.2 protein subsequently was immunoprecipitated and analyzed as described above.

GST fusion binding analysis

DNA encoding the N-terminal domain (amino acids 1–163) and C-terminal domain (amino acids 426–499) of Kv1.2 was generated by PCR and fused in-frame to the GST coding sequence in pGEX-2T. Fusion proteins were expressed in *Escherichia coli* BL21 (DE3) or TKB1 cells, induced with 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) and prepared according to the method described by Grieco *et al.* (1992). The solubilized fusion proteins were purified by incubation with glutathione-conjugated agarose beads at room temperature for 10 min and washed three times with 200 vols of phosphate-buffered saline (PBS), 0.01% Triton X-100. Approximately 5 μ g of purified GST fusion protein bound to agarose beads were incubated for 5–8 h at 4°C with lysates from m1K-293 cells which had been treated with or without carbachol for 30 min at 37°C. The beads subsequently were washed twice with RIPA buffer. Bound proteins were eluted from the beads by heating in protein sample buffer at 100°C and then separated by SDS-PAGE. RPTP α or RPTP β proteins were detected by immunoblotting with an RPTP α - or RPTP β -specific antibody. GST fusion proteins were visualized by Coomassie staining.

Electrophysiology

Whole-cell patch-clamp recordings were taken from m1K-293 cells. The cells were plated at low density on poly-D-lysine-coated glass coverslips. To prevent space clamp artifacts through gap junctions, all recordings were taken from individual cells that were not in contact with other cells. Data were collected using an Axopatch 200 patch-clamp interfaced to a Gateway 2000 486 computer using pCLAMP V6 (Axon Instruments). Recording pipettes (VWR 100 μ l) were pulled to a resistance of 1 M Ω . All traces were low pass filtered at 1 kHz and leak subtracted using a P/8 protocol from a holding potential of –60 mV. The pipette solution contained 60 mM K₂SO₄, 1.2 mM KCl, 5 mM MgSO₄, 5 mM Na⁺-HEPES and 35 mM sucrose (pH 7.1). The external (bath) solution contained 118 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 5 mM Na⁺-HEPES and 23 mM glucose (pH 7.4). Tetraethylammonium chloride (TEA) was included in the bath (20 mM) to block endogenous potassium currents. For the indicated experiments, 1 mM vanadate was also included in the external saline. Cells expressing GFP or RPTP α were tested 48 h after transfection. Two-electrode voltage clamp (Warner) was used to measure the current from individual *Xenopus* oocytes. RNAs encoding RPTP α and Kv1.2 were injected to a concentration of 5 pg per oocyte. Recordings were made at room temperature in Barth's solution 2 days after RNA injection. Current and potential recording electrodes were pulled to a resistance of 0.5–1 M Ω and filled with 3 M KCl. Recordings and stimulations were controlled using pClamp (Axon Instruments).

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