Modulation of AMPA/kainate receptors in cultured murine hippocampal neurones by protein kinase C

Lu-Yang Wang*, E. M. Dudek†, M. D. Browning† and J. F. MacDonald*‡

*Departments of Physiology and Pharmacology, University of Toronto, Medical Sciences Building, Toronto, Ontario, Canada M5S 1A8 and †Department of Pharmacology, Program in Neuroscience, University of Colorado Health Science Center, University of Colorado, Denver, CO 80262, USA

1. The patch clamp technique, together with intracellular perfusion of the catalytic fragment of protein kinase C (PKCM), was employed to investigate the role of this enzyme in the intracellular regulation of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/kainate receptors in cultured hippocampal neurones.

2. The responses evoked by near-maximal concentrations of kainate (250 μM) and AMPA (100 μM) were potentiated by the introduction of PKCM, whilst co-application of the inhibitory peptide fragment PKCI(19–36) prevented this action.

3. Modulation of kainate responses by PKCM was dependent upon the concentration of agonist applied. Currents evoked by kainate were potentiated at concentrations above those which caused 50% of the maximal response (EC_{50}) and depressed at lower concentrations. Furthermore, okadaic acid, a specific inhibitor of phosphatases 1 and 2A, had a similar effect upon concentration–response relationships when currents activated by kainate were recorded using the perforated patch technique.

4. In addition, the mean amplitude and/or time constant of decay of miniature excitatory synaptic currents (mediated by AMPA/kainate receptors) was increased by the intracellular injection of PKCM.

5. These observations suggest that the function of postsynaptic excitatory amino acid receptors can be modulated by the activity of PKC as well as by endogenous phosphatases. This regulation may contribute to some forms of synaptic plasticity within the central nervous system.

Excitatory synapses in the hippocampus and various other brain regions undergo a long-lasting increase in their efficacy, termed long-term potentiation (LTP). However, the mechanisms underlying the maintenance of LTP and/or the site of the persistent change in synaptic efficacy have been the subject of intensive debate. Measurements of the release of endogenous glutamate and quantal analysis suggest that there is a maintained enhancement of presynaptic release during LTP. In contrast, other experimental results favour a postsynaptic modification of receptor sensitivity. For example, postsynaptic responses to exogenously applied AMPA increase progressively during LTP (Davies, Lester, Reymann & Collingridge, 1989). Thus, both presynaptic and postsynaptic mechanisms may contribute to LTP (Bliss & Collingridge, 1993).

An influx of Ca^{2+} through NMDA channels plays a critical role in the induction of LTP. In the dendritic spines of postsynaptic neurones the resulting increases in the concentration of intracellular Ca^{2+} can activate protein kinases, primarily Ca^{2+}- and phospholipid-dependent protein kinase C (PKC) and Ca^{2+}–calmodulin kinase II (CaMKII; Bliss & Collingridge, 1993). Proteolytic cleavage of PKC by proteases such as calpain converts it to a constitutively active form (Klann, Chen & Sweatt, 1993) whereas CaMKII is converted into a Ca^{2+}/calmodulin-independent form by autophosphorylation. Glutamate receptor proteins are potential substrates for these kinases, suggesting that their phosphorylation could contribute to LTP by either increasing their sensitivity to the transmitter or increasing the number of functional receptors. We have therefore examined the possibility that PKC modulates AMPA/kainate receptors in cultured hippocampal neurones. This category of glutamate receptors mediate most of the postsynaptic response in hippocampal neurones (Bliss & Collingridge, 1993). Part of this report has appeared in abstract form (Wang, Dudek, Browning & MacDonald, 1992).

‡To whom correspondence should be addressed.

METHODS

Monolayer cultures of hippocampal neurones were grown as previously described (MacDonald, Mody & Salter, 1989) and used for patch clamp recording (Axopatch-1B, Axon Instruments, Foster City, CA, USA) 14–21 days after plating. Mice were killed by cervical dislocation. In order to record whole-cell currents or miniature excitatory postsynaptic currents (mEPSCs), the electrodes were filled with (mM): 140 KCl, 10 Hepes, 6 $MgCl_2$, 5 EGTA, 2 tetraethylammonium, 1 $CaCl_2$, 4 K^+-ATP (pH 7·5). In some experiments perforated patch recordings were made using nystatin as previously described (Wang, Salter & MacDonald, 1991). The extracellular solution contained (mM): 140 NaCl, 1·3 $CaCl_2$, 5·4 KCl, 25 Hepes, 33 glucose, 0·003 glycine and 0·001 tetrodotoxin (pH 7·4, 320–335 mosmol l^{-1}). Bicuculline (20 μM), $MgCl_2$ (1 mM) and 2-amino-5-phosphonovaleric acid (20 μM) were also included for recordings of mEPSCs. Data were digitized, filtered (2 kHz) and acquired on-line using pCLAMP (Axon Instruments) or Scan (Strathclyde Electrophysiology Software, courtesy of Dr J. Dempster) and stored on tape for further analysis. Statistical significance was assessed using Student's two-tailed t test. Amino acids and okadaic acid were applied through a three-barrelled perfusion system.

Protein kinase C was purified with slight modification of published procedures (Roth, Mehegan, Jacobowitz, Robey & Iadarola, 1989) using sequential chromatography on diethyl aminoethyl (DEAE)-cellulose, phenyl sepharose and protamine agarose. The catalytically active fragment of PKC was prepared by digestion with trypsin as described by Huang & Huang (1986). After trypsinization the 45 kDa catalytic fragment (PKCM) was repurified by chromatography on DEAE-52 or by soy bean trypsin inhibitor affinity chromatography. The PKCM fractions were pooled, concentrated and dialysed extensively against the recording buffer. The samples were then frozen in small aliquots and thawed just before use. The specific activity of the PKCM was 1–2 μmol min^{-1} mg^{-1} using Histone III-S (Sigma, St Louis, MO, USA) as the substrate. The concentration of the kinase in the perfusion pipette was 4 μM. PKCI (PKC(19–36), RFARKGALRQKNVHEVKN) was made on a peptide synthesizer (model 430A, Applied Biosystems, Foster City CA, USA) and was purified by reverse-phase chromatography on a fast protein liquid chromatography (FPLC) machine (Pharmacia, Piscataway, NJ, USA) using acetonitrile gradient elution. The acetonitrile was removed by three cycles of lyophilization and the peptide was aliquoted into microfuge tubes and frozen until just before use. The aliquots were

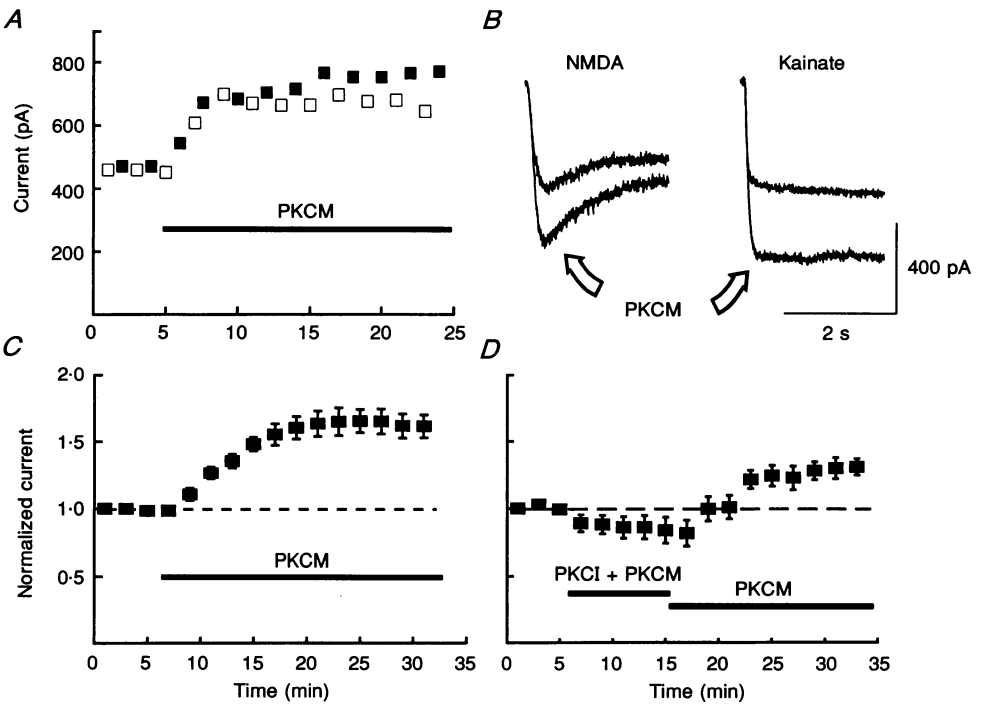

Figure 1. The catalytic fragment of PKC enhances both NMDA and kainate currents
A, whole-cell recordings of peak NMDA (250 μM, □) and kainate (250 μM, ■) currents prior to and during the internal perfusion of PKCM (4 μM in the pipette). NMDA currents were significantly potentiated by PKCM (140·9 ± 3·6 %, $n = 6$). *B*, representative current traces before and after PKCM injection. *C*, mean data (± S.E.M.) from a series of recordings of kainate currents. The maximum potentiation was 165·3 ± 8·7 % ($n = 7$). In several cells with very stable recordings the potentiation was maintained for as long as 1 h. *D*, in contrast a mixture of PKCI (10 μM) and PKCM (4 μM) not only prevented this potentiation but also inhibited control currents (to 81·8 ± 9·6 %, $n = 5$). Potentiation could still be achieved following the subsequent injection of PKCM alone (130·6 ± 6·2 %). Bars in *A*, *C* and *D* indicate the onset, offset and the duration of the internal perfusion. The holding potential in this and subsequent figures was −60 mV, except where otherwise indicated.

reconstituted in the recording buffer. In whole-cell intracellular perfusion studies PKCI was used at a final concentration of 10 μM.

RESULTS

Enhancement of macroscopic NMDA and kainate currents by PKCM

Since the currents evoked by glutamate and AMPA in cultured hippocampal neurones display a rapid and substantial desensitization while those evoked in response to kainate do not, we have primarily employed kainate to activate AMPA/kainate receptors. In order to reduce the run-down of these currents we have also included adenosine-5-triphosphate (ATP) in the recording pipettes (MacDonald *et al.* 1989; Wang *et al.* 1991). Stable control currents were first established in each recording and then the catalytic fragment (PKCM) was introduced into the neurone via a second pipette placed inside the patch electrode. Unlike the holoenzyme itself, the activity of this fragment is entirely independent of calcium and phosphatidylserine.

It has been reported that PKC selectively potentiates currents activated by NMDA in hippocampal neurones without affecting kainate or AMPA currents (Ben-Ari, Aniksztejn & Bregestovski, 1992). In contrast, we observed that PKCM consistently increased both NMDA- and kainate-evoked currents recorded from the same cultured hippocampal cell (Fig. 1*A* and *B*).

Kainate currents were maximally potentiated to 165% of control and this value was reached in about 15 min following the onset of the perfusion of PKCM (Fig. 1*C*). To assess the specificity of this effect we also co-perfused PKCM and PKCI(19–36). The latter combination did not potentiate the currents, which were instead inhibited to about 82% of the control level (Fig. 1*D*), suggesting a possible regulation of the receptors by endogenous PKC. Subsequent injection of PKCM reversed this inhibition and subsequently potentiated kainate currents to 130% of control. In a separate series of recordings it was shown that the slope of the current–voltage relationship in the presence of kainate was increased by PKCM, but there was no change in the reversal potential (Fig. 2*A* and *B*). In some experiments neurones were pretreated with wheat-germ agglutinin in order to block receptor desensitization (Wang, Taverna, Huang, MacDonald & Hampson, 1993) and the effects of PKCM on currents evoked by applications of AMPA were examined. Under these conditions the intracellular perfusion of PKCM caused significant potentiation of these currents (Fig. 2*C*).

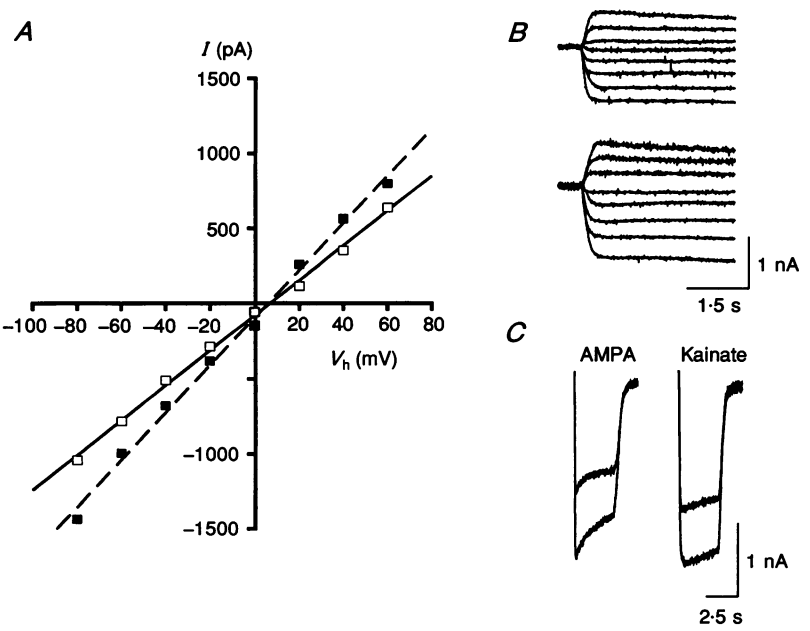

Figure 2. PKCM increases the whole-cell conductance of kainate-activated currents
A, current–voltage relationships determined before (□) and after (■) the application of PKCM. The holding potential (V_h) was varied from −80 to +60 mV and kainate (250 μM) was applied once at each holding potential. *B*, individual kainate currents at each holding potential (leak subtracted) before (upper trace) and after (lower trace) the injection of PKCM. *C*, a typical recording of AMPA (100 μM, left trace) and kainate (250 μM, right trace) currents from a wheat-germ agglutinin (10 μg ml^{-1}) pretreated neurone showing a similar degree of enhancement of both currents by PKCM. The mean potentiation was 151·8 ± 8·5% (n = 3) for AMPA.

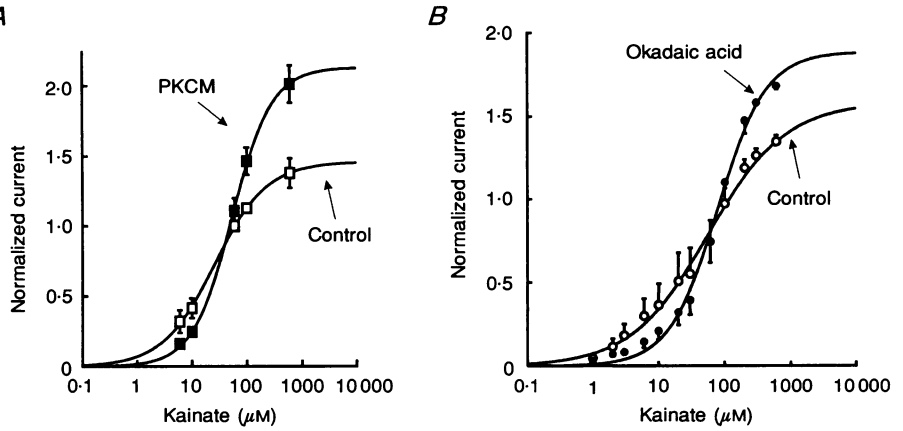

Figure 3. Intracellular PKCM and treatment with the phosphatase inhibitor okadaic acid have similar actions on kainate concentration–response curves

Dose–response relationships determined before (open symbols) and after (filled symbols) the application of PKCM (*A*) or okadaic acid (*B*; 250 nM). Individual dose–response curves were fitted using the logistic equation in the form: $I = I_{max}/(1 + (d/EC_{50})^{n_H})$, where I represents the calculated current amplitude at any given kainate concentration (d), I_{max} is the response to a saturating concentration of agonist, EC_{50} is the concentration of agonist that produces 50 % of the maximal response and n_H is the estimated Hill coefficient. Paired dose–response curves recorded in individual cells were normalized to the current amplitude at 60 μM kainate prior to the injection of PKCM. *A*, I_{max}, n_H and EC_{50} were 1·51 ± 0·12, 1·08 ± 0·28 and 28·98 ± 6·02 μM, respectively for controls; 2·14 ± 0·14*, 1·20 ± 0·05 and 55·22 ± 4·66 μM* respectively for PKCM ($n = 5$). *B*, I_{max}, n_H and EC_{50} were 1·51 ± 0·08, 1·01 ± 0·14 and 43·7 ± 17·9 μM, respectively for controls; 1·83 ± 0·06*, 1·18 ± 0·13, 70·6 ± 20·6 μM*, respectively for okadaic acid ($n = 4$) (*$P < 0·05$).

Modulation of kainate currents by PKCM and okadaic acid depends upon the concentration of agonist

We also examined concentration–response relationships for kainate, before and following injection of PKCM (Fig. 3*A*). The EC_{50} values, maximal responses and Hill slope coefficients (n_H) were then estimated. The modulation of kainate currents that followed injections of PKCM was strongly dependent upon the concentration of agonist applied being potentiated at concentrations required to evoke maximal responses and depressed at low concentrations. The EC_{50} value for kainate was significantly increased by PKCM, suggesting a reduction in the apparent affinity.

We and others have previously reported that AMPA/kainate receptors are modulated by protein phosphatases 1 and 2A (Wang *et al.* 1991; McGlade-McCulloh, Yamamoto, Tan, Brickey & Soderling, 1993). To explore further the functional consequence of inhibition of endogenous phosphatases, concentration–response relationships were constructed in perforated patches before and after application of the membrane-permeable phosphatase inhibitor okadaic acid. Perforated patches were employed in order to minimize the disruption of endogenous kinase activity and to prevent the run-down of glutamate currents which would have occurred during whole-cell recording. The effects of okadaic acid on kainate currents were also similarly dependent upon the agonist concentration (Fig. 3*B*). In addition, there was also a shift to a lower apparent affinity.

Postsynaptic facilitation of excitatory neurotransmission

Since the mEPSC probably represents the release of a single quantum of transmitter, analysis of such currents can help to distinguish changes in quantal size from alterations in the probability of release. Therefore, we evoked mEPSCs using repeated applications (2 s) of hypertonic solution (sucrose, 0·5 M; from a pipette placed less than 10 μm from the soma) under conditions which blocked NMDA receptors. In each recording a number (50–200) of consecutive mEPSCs were averaged prior to and after the intracellular perfusion of PKCM. These currents typically displayed a rapid rise to peak followed by an exponential phase of decay (τ about 2–3 ms) and they were entirely blocked by the AMPA/kainate receptor antagonist CNQX (6-cyano-7-nitroquinoxaline-2,3-dione; 5 μM). In eleven of fourteen neurones we observed a significant increase in the time constant of decay (before $\tau = 2·77 \pm 0·22$ ms; after $\tau = 3·92 \pm 0·23$ ms, $P < 0·05$; Fig. 4*A*) of mEPSCs following application of PKCM. In the remaining three cells and two of the aforementioned eleven cells, an increase in peak mEPSC amplitude (15·2 ± 2·4 to 20·1 ± 3·8 pA, $P < 0·05$, Fig. 4*B*) was also observed. We detected concurrent increases in the amplitude of both whole-cell currents and spontaneous mEPSCs in three cells following the injection of PKCM (Fig. 4*C* and *D*).

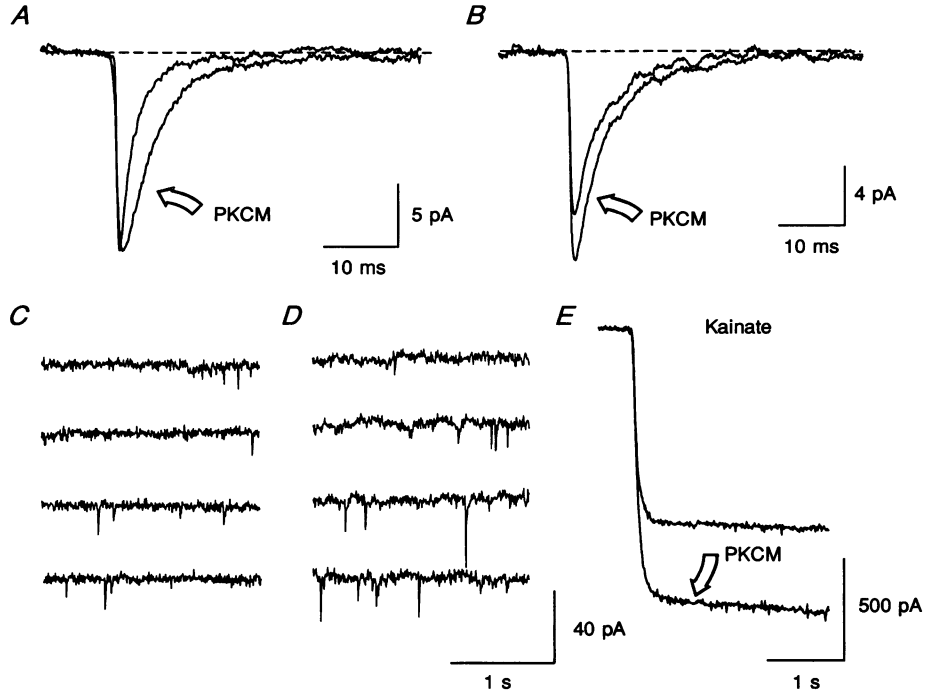

Figure 4. PKCM enhances minature synaptic currents
Examples from two different cells showed that PKCM increased either the averaged amplitude (*A*, increased from 10·8 to 14·4 pA) or the averaged time constant of decay (*B*, τ increased from 2·4 to 4·4 ms) of sucrose-evoked mEPSCs. The averaged traces before and after intracellular perfusion of PKCM are superimposed in *A* and *B*. Each trace represents the mean of 200 (*A*) or 76 (*B*) consecutive mEPSCs. A recording from another cell illustrates that there was also an increase in the amplitude of spontaneous mEPSCs (*C*, control, mean amplitude 9·2 pA; *D*, after PKCM, mean amplitude 15·8 pA). The final panel (*E*) illustrates that kainate currents (250 μM) in this cell were also enhanced (control, 941·8 pA; PKCM, 1336·5 pA).

DISCUSSION

Our results demonstrate that AMPA/kainate receptors expressed in cultured hippocampal neurones can be modulated by the activity of PKC. The intracellular perfusion of PKCM into these neurones consistently potentiated the currents activated by relatively high concentrations of kainate (e.g. 250 μM). On the other hand, co-perfusion of PKCM with the inhibitory peptide PKCI completely prevented this potentiation, confirming the selective role of this kinase in the observed effect. The injection of PKCI also produced an inhibition to approximately 82% of the control currents, suggesting that PKCI may have inhibited endogenous kinase activity responsible for regulating receptor function. This depression of kainate responses by PKCI probably resulted from the inhibition of endogenous PKC ($IC_{50} = 0{\cdot}3\ \mu$M) although a partial inhibition of CaMKII ($IC_{50} = 4{\cdot}9\ \mu$M; Smith, Colbran & Soderling, 1990) may also have contributed. Both kinases can phosphorylate at least one subtype of AMPA/kainate receptor found in the postsynaptic densities of cultured hippocampal neurones (McGlade-McCulloh *et al.* 1993). In contrast, cAMP-dependent protein kinase (PKA; $IC_{50} > 60\ \mu$M) is unaffected by this PKCI (Smith *et al.* 1990).

The modulation of AMPA/kainate receptors by PKCM was complex. Potentiation of kainate (and AMPA) currents was only observed at concentrations well above that of the EC_{50} value. This observation may account for the previous reports of a lack of effect of PKC on AMPA/kainate receptors (Ben-Ari *et al.* 1992). The increase in the maximal response following injection of PKCM suggests that phosphorylation may have increased the number of active receptors, as has been suggested for the effects of PKA on acetylcholine receptors in chick cilary ganglion neurones (Margiotta, Berg & Dionne, 1987). However, PKCM also increased the apparent dissociation constant (EC_{50} value) for kainate and a modest reduction in amplitude of these currents was observed when low concentrations of agonist were applied. This evidence suggests that phosphorylation may have changed the affinity of the receptors for kainate or, alternatively, it may have revealed the presence of two categories of receptors, each of which is modulated differently by PKC. Modulation of currents evoked by kainate need not have arisen either in its entirety or in part from the direct phosphorylation of receptor proteins.

For example, PKC may have increased the activity of other protein kinases such as PKA or CaMKII, which in turn may have modulated this channel activity (Browning & Dudek, 1992). Alternatively, PKC may have influenced the aggregation and assembly of AMPA/kainate subunits as discussed by Swope, Moss, Blackstone & Huganir (1992). In addition, many cytoskeletal proteins, including actin and myosin light chain protein, are substrates for phosphorylation by PKC and phosphorylation of these proteins modulates the integrity of the cytoskeleton (Eriksson, Brautigan, Vallee, Olmsted, Fujiki & Goldman, 1992). In turn, changes in the polymerization state of actin filaments induced by phosphorylation can have profound effects upon channel activity (Prat, Bertorello, Ausiello & Cantiello, 1993).

We have shown that in cultured hippocampal neurones the currents evoked by applications of kainate or AMPA can be modulated by a constitutively active form of PKC. In addition, the intracellular perfusion of PKCM increased the amplitude and/or time constant of decay of mEPSCs. It should be noted that drugs which increase the time constant of decay of the AMPA/kainate mEPSC can also increase the amplitude of evoked EPSCs (Vyklický, Patneau & Mayer, 1991). As PKCM only caused an enhancement of AMPA/kainate currents when concentrations of agonist considerably greater than the EC_{50} value were employed it might be argued that presynaptically released L-glutamate must reach near-saturating concentrations at the postsynaptic receptors for phosphorylation to play a role in modulating synaptic transmission. Indeed, recent estimates of the concentration of glutamate achieved at the synapses in cultured neurones are as high as 1 mM (Clements, Lester, Tong, Jahr & Westbrook, 1992).

Molecular cloning of AMPA/kainate and kainate receptors has shown that the intracellular loop between third and fourth transmembrane domains is highly conserved and contains consensus sequences for both PKC and CaMKII kinase (Swope *et al.* 1992), suggesting that they may be substrates for phosphorylation by PKC. Recent *in vitro* biochemical experiments have indicated that GluR1, one of the subunits expressed in cultured hippocampal neurones, is phosphorylated by both PKC and CaMKII kinase (McGlade-McCulloh *et al.* 1993). Phosphorylation of this subunit by PKC may have contributed to the modulation of AMPA/kainate receptors observed by us.

There is strong evidence that LTP involves changes in both pre- and postsynaptic components of glutamatergic transmission in area CA1 of the hippocampus and potentiation of AMPA/kainate receptor function may underlie, at least in part, the postsynaptic potentiation seen with LTP (Bliss & Collingridge, 1993). The formation of a constituently active form of PKC appears to be required for the mainenance of LTP (Sacktor, Osten, Valsamis, Jiang, Naik & Sublette, 1993). Our results suggest that AMPA/kainate receptors in hippocampal neurones can be functionally regulated by PKC phosphorylation and that this mechanism may contribute to this and perhaps other forms of synaptic plasticity in the central nervous system.

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