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# A new signalling pathway for parallel fibre presynaptic type 4 metabotropic glutamate receptors (mGluR4) in the rat cerebellar cortex

Karine Abitbol, Heather McLean, Thomas Bessiron and Hervé Daniel

*Pharmacology and Biochemistry of the synapse, CNRS UMR 8619, Institut de Biochimie et de Biophysique Moléculaire et Cellulaire, Université Paris-Sud, 91405 Orsay Cedex, France*

**Key points**

- Glutamate is the major excitatory neurotransmitter of the mammalian brain. It can activate ionotropic receptors underlying fast glutamatergic transmission as well as G protein-coupled metabotropic receptors (mGluR1–8), which are pre- and postsynaptic modulators of this fast excitatory neurotransmission.
- In the rodent cerebellum, activation of presynaptic mGluR4 depresses excitatory synaptic transmission at parallel fibre–Purkinje cell synapses. We show that this depression involves the inhibition of presynaptic calcium influx via a newly defined signalling pathway, which notably involves the activation of phospholipase C and ultimately protein kinase C.
- The study of the molecular basis of mGluR signalling pathways is an important research topic because these receptors may be implicated in certain neurodegenerative disorders, like Parkinson's or Alzheimer's disease. As such, these receptors are becoming crucial targets for a number of therapeutic agents.

**Abstract** In the rodent cerebellum, pharmacological activation of mGluR4 acutely depresses excitatory synaptic transmission at parallel fibre–Purkinje cell synapses. This depression involves the inhibition of presynaptic calcium ($Ca^{2+}$) influx that ultimately controls glutamate release. In this study, we investigate the molecular basis of mGluR4-mediated inhibition of presynaptic $Ca^{2+}$ transients. Our results demonstrate that the mGluR4 effect does not depend on selective inhibition of a specific type of presynaptic voltage-gated $Ca^{2+}$ channel, but rather involves modulation of all classes of $Ca^{2+}$ channels present in the presynaptic terminals. In addition, this inhibitory effect does not involve the activation of G protein-activated inwardly rectifying potassium channels, TEA-sensitive potassium channels or two-pore-domain potassium channels. Furthermore, this inhibition does not require pertussis toxin-sensitive G proteins, and is independent of any effect on adenylyl cyclases, protein kinase A, mitogen-activated protein kinases or phosphoinositol-3 kinase activity. Interestingly we found that mGluR4 inhibition of presynaptic $Ca^{2+}$ influx employs a newly defined signalling pathway, notably that involving the activation of phospholipase C and ultimately protein kinase C.

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**Corresponding author** H. Daniel: Laboratoire de Pharmacologie et Biochimie de la synapse, CNRS UMR 8619, Institut de Biochimie et de Biophysique Moléculaire et Cellulaire, Université Paris-Sud XI, 91405 Orsay Cedex, France. Email: herve.daniel@u-psud.fr



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**Abbreviations** AC, adenylyl cyclase; DEA/NO, diethylamine NONOate sodium salt hydrate; GIRK, G protein-gated inwardly rectifying $K^+$ channels; GPCR, G protein-coupled receptor; K2P, two-pore domain $K^+$ channel; LTD, long-term depression; LTP, long-term potentiation; MAPK, mitogen-activated protein kinase; mGluR, metabotropic glutamate receptor; PC, Purkinje cell; PF, parallel fibre; PLC, phospholipase C; PPF, paired-pulse facilitation; PTX, pertussis toxin; VGCC, voltage-gated calcium channel.

## Introduction

In the rodent cerebellum, group III metabotropic glutamate receptors (mGluRs) negatively regulate $Ca^{2+}$ influx into presynaptic terminals (Daniel & Crepel, 2001; Zhang & Linden, 2009), and thus decrease glutamatergic transmission (Conquet *et al*. 1994; Pekhletski *et al*. 1996; Miniaci *et al*. 2001; Neale *et al*. 2001; Lorez *et al*. 2003). We have shown that among the group III mGluRs present at the parallel fibre (PF)–Purkinje cell (PC) synapse, presynaptic mGluR4s are entirely responsible for this regulation (Abitbol *et al*. 2008).

In PF terminals, $Ca^{2+}$ influx results from the activation of diverse $Ca^{2+}$ channels, notably P/Q-, N- and R-type channels (Mintz *et al*. 1995; Brown *et al*. 2004; Daniel *et al*. 2004). At many synapses, group III mGluRs depress neurotransmitter release by inhibiting one or more of these voltage-gated $Ca^{2+}$ channels (VGCCs). For example, group III mGluR activation inhibits P/Q-type channels in the superior olivary complex (Takahashi *et al*. 1996), N-type channels in the hippocampus (Capogna, 2004; Rusakov *et al*. 2004), and both N- and P/Q-type channels in the entorhinal cortex (Woodhall *et al*. 2007) and in cerebrocortical synaptosomes (Millan *et al*. 2002). In contrast, group III mGluR-mediated presynaptic inhibition in cultured reticulospinal neurons does not involve selective modulation of any known type of presynaptic VGCC (Krieger *et al*. 1999).

mGluRs may also have indirect effects on VGCC activity by modulating ionic channels that control presynaptic membrane excitability. Candidate channels include: 4-aminopyridine (4-AP)-sensitive $K^+$ channels (Daniel & Crepel, 2001) and G protein-gated inwardly rectifying $K^+$ channels (GIRKs) (Saugstad *et al*. 1996; Sharon *et al*. 1997). In addition, certain subfamilies of two-pore-domain $K^+$ channels (K2P) (Honoré, 2007), including TREK and TASK, are present on cerebellar granule cells (Watkins & Mathie 1996; Talley *et al*. 2001) and are regulated by G protein-coupled receptors (GPCRs) (Mathie, 2007), including mGluR4 (Cain *et al*. 2008).

While many group III mGluRs act through pertussis toxin (PTX)-sensitive $G_{i/o}$ proteins that inhibit adenylyl cyclase (AC) activity and decrease intracellular cAMP levels (Thomsen *et al*. 1992; Kristensen *et al*. 1993; Tanabe *et al*. 1993; Prezeau *et al*. 1994; Flor *et al*. 1995; Neil *et al*. 1996; Conn & Pin, 1997), several studies show alternative signalling pathways for these receptors. For example, they can stimulate AC activity leading to increases in cAMP levels (Lavialle-Defaix *et al*. 2006) and activation of protein kinase A (PKA) (Evans *et al*. 2001), probably through $G_s$ proteins. In cultured cerebellar granule cells, native group III mGluRs are functionally coupled to both mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) pathways (Iacovelli *et al*. 2002). Finally, within this group, mGluR7 inhibits P/Q-type VGCCs through the phospholipase C (PLC)–protein kinase C (PKC) cascade (Perroy *et al*. 2000).

We used fluorometric and electrophysiological approaches to investigate mGluR4-mediated inhibition of evoked presynaptic $Ca^{2+}$ influx. We show that this modulation does not selectively target a specific type of presynaptic VGCC and is independent of any modulation of presynaptic $K^+$ channels, MAPK or PI3K activity, or the $G_{i/o}$–AC–PKA signalling pathway. Interestingly we provide evidence suggesting that the activation of PF terminal mGluR4s initiates a non-canonical intracellular cascade that involves PLC and PKC.

## Methods

### Preparation of cerebellar slices

Animal care and all experimental procedures are in accordance with guidelines from the Centre National de la Recherche Scientifique (CNRS, France). Male Sprague–Dawley rats, 18–34 days old, were stunned and then decapitated. Coronal or sagittal cerebellar slices (200–250 $\mu$m thick) were prepared as previously described (Daniel & Crepel, 2001). The slices were kept at room temperature in saline solution gassed with 95% $O_2$–5% $CO_2$ for at least 1 h before recording. This solution contained (in mM): NaCl, 124; KCl, 3; $NaHCO_3$, 24; $KH_2PO_4$, 1.15; $MgSO_4$, 1.15; $CaCl_2$, 2; glucose, 10 and registered an osmolarity of 330 mosmol $l^{-1}$ and a pH of 7.35 at 25°C. The recording chamber was perfused at a rate of 2 ml per minute with this same oxygenated saline solution, supplemented with the $GABA_A$ receptor antagonist bicuculline methochloride or methiodide (10 $\mu$M, Sigma Aldrich, St Quentin Fallavier, France) to block inhibitory membrane currents mediated by these receptors.

### Pharmacological agents

Most pharmacological agents were applied to cerebellar slices by direct addition to the saline solution, with

the exception of pertussis toxin (PTX), KT 5720, U-73122, U-73343 and Ro 32-0432 with which the slices were incubated in the saline solution for variable durations before the recording session. In some experiments, slices were pretreated with thapsigargin (10 $\mu$M) for 40 min. L-AP4, PTX, KT 5720, Tertiapin Q, U-73122, Ro 32-0432, LY 294002, DHPG, PD98059, WIN55,212-2, NBQX, D-AP5 and phorbol 12 -Myristate 13 - Acetate (PMA) were purchased from Tocris (Illkirch, France). SNX-482, $\omega$-agatoxin TK, $\omega$-conotoxin GVIA and thapsigargin were purchased from Alomone Labs (Israel). 2′,5′-dideoxyadenosine, SQ 22,536, 4-aminopyridine (4-AP), U-73343, ruthenium red, cadmium, TEA and DEA/NO were obtained from Sigma. Fluoxetine was purchased from Ascent Scientific. All drug stocks were prepared in distilled water, except stocks of Fluo-4FF AM (Molecular Probes), 2′,5′-dideoxyadenosine, KT 5720, PD98059, LY 294002, PMA, U-73122, U-73343, SQ 22,536 and Ro 32-0432 which were prepared in dimethylsulfoxide (DMSO). Drug stocks were kept at −20°C until the day of the experiment. Unless otherwise stated, drugs were added to the perfusate at the desired concentration just before application (final concentration of DMSO was 0.1%).

## Electrophysiology

Whole-cell patch-clamp recordings of Purkinje cell (PC) somas were performed in sagittal slices with an Axopatch-1D amplifier (Axon Instruments). All recordings were made at 27–28°C. Patch pipettes (3.5–5 MΩ, borosilicate glass) were filled with an internal solution of the following composition (mM): Nacl, 140; KCl, 6; Hepes, 10; EGTA, 0.75; $MgCl_2$, 1; Na-GTP, 0.4; $Na_2$-ATP, 4; pH 7.3 with KOH; 300 milliosmol $l^{-1}$ As previously reported (Goossens *et al*. 2001), PCs were clamped at −70 mV (junction potentials corrected) and parallel fibres (PFs) were stimulated once every 6 s through an extracellular glass saline-filled monopolar electrode placed at the surface of the slice, in the lower half of the molecular layer, to evoke PF-mediated excitatory postsynaptic currents (EPSCs). Recorded PF-EPSCs were filtered at 5 kHz, digitized on line at 20 kHz, and analysed on- and off-line with Acquis1 software (Biologic, Grenoble, France). In the cells conserved for analysis, access resistance (usually 5–10 MΩ) was partially compensated (50–70%), according to the procedure described by Llano *et al*. (1991). Throughout the experiment, PF-EPSCs were elicited on a 10 mV hyperpolarizing voltage step, which allowed monitoring of passive membrane properties of the recorded cells.

In some experiments, PF-EPSCs were evoked with pairs of stimuli of the same intensity applied to the cell with an inter-stimulus interval of 40 ms. Paired-pulse facilitation (PPF) values (Atluri & Regehr, 1996) were calculated on-line as the ratio of the amplitude of the second PF-EPSC over the first one and plotted against time. Corresponding PPF values in individual plots were then averaged for all cells recorded to obtain the plot of mean PPF values before, during and after bath application of the mGluR4 agonist, L-AP4.

The PF volley is an extracellular field potential that was recorded with a saline-filled glass microelectrode placed in the molecular layer 500–800 $\mu$m away from the stimulus site. To prevent contamination by excitatory post-synaptic signals, PF volley experiments were performed in the presence of 20 $\mu$M NBQX and 50 $\mu$M D-AP5, respective antagonists of AMPA–kainate and NMDA receptors.

## Calcium-sensitive fluorometric measurements

Using coronal slices, presynaptic PF tracts were labelled by local application of a saline solution containing the low-affinity calcium indicator Fluo-4FF AM (100 $\mu$M), as previously described (Daniel & Crepel, 2001). At least 30 min after loading, a confined region of labelled PFs was illuminated at a single excitation wavelength (480 ± 22 nm). Excitation light obtained from a 100 W mercury lamp was gated with an electromechanical shutter (Uniblitz, Rochester, NY, USA). The optical signals were recorded at 27–28°C, through a 20 $\mu$m × 50 $\mu$m window placed in the molecular layer on the visible narrow band of labelled PFs, approximately 500–700 $\mu$m away from the loading site. At this distance, only the loaded fibre tracts were visible in the recording window; no other labelled structures were detectable. PFs located in the recording window were stimulated every 30 s with a single 100 Hz train of five electrical stimuli, through a saline-filled glass microelectrode placed in the molecular layer between the loading site and the recording site. Stimulation-evoked $Ca^{2+}$-sensitive changes in fluorescence were acquired through a ×60 water-immersion objective of an upright microscope (Zeiss, LePeck France), filtered by a barrier filter at 530 ± 30 nm and converted into an electric signal by a photometer. Fluorometric measurements were analysed on- and off-line using Acquis1 software. The fluorescence data corrected for dye bleaching were expressed as relative fluorescence changes $\Delta F/F$, where $F$ is the baseline fluorescence intensity, and $\Delta F$ is the change induced by PF stimulation. When background fluorescence of the tissue in unlabelled regions of the slice was greater than 5% of the resting fluorescence intensity of the indicator, the data were corrected for background fluorescence. Statistical significance was assessed by an unpaired Student's $t$ test, with $P < 0.05$ (two-tailed) considered as significant. All data are expressed as the mean ± SEM.

## Results

The presynaptic molecular events associated with pharmacological activation of mGluR4s were explored in coronal rat cerebellar slices with fluorometric methods, using the low-affinity $Ca^{2+}$-sensitive dye Fluo-4FF AM, which allows a linear measure of presynaptic $Ca^{2+}$ influx. As shown in our previous study (Abitbol *et al.* 2008), a train of five stimulations applied to PFs induced reproducible transient increases in presynaptic fluorescence, which returned to resting levels within a few hundred milliseconds (Fig. 1*A*, inset). In keeping

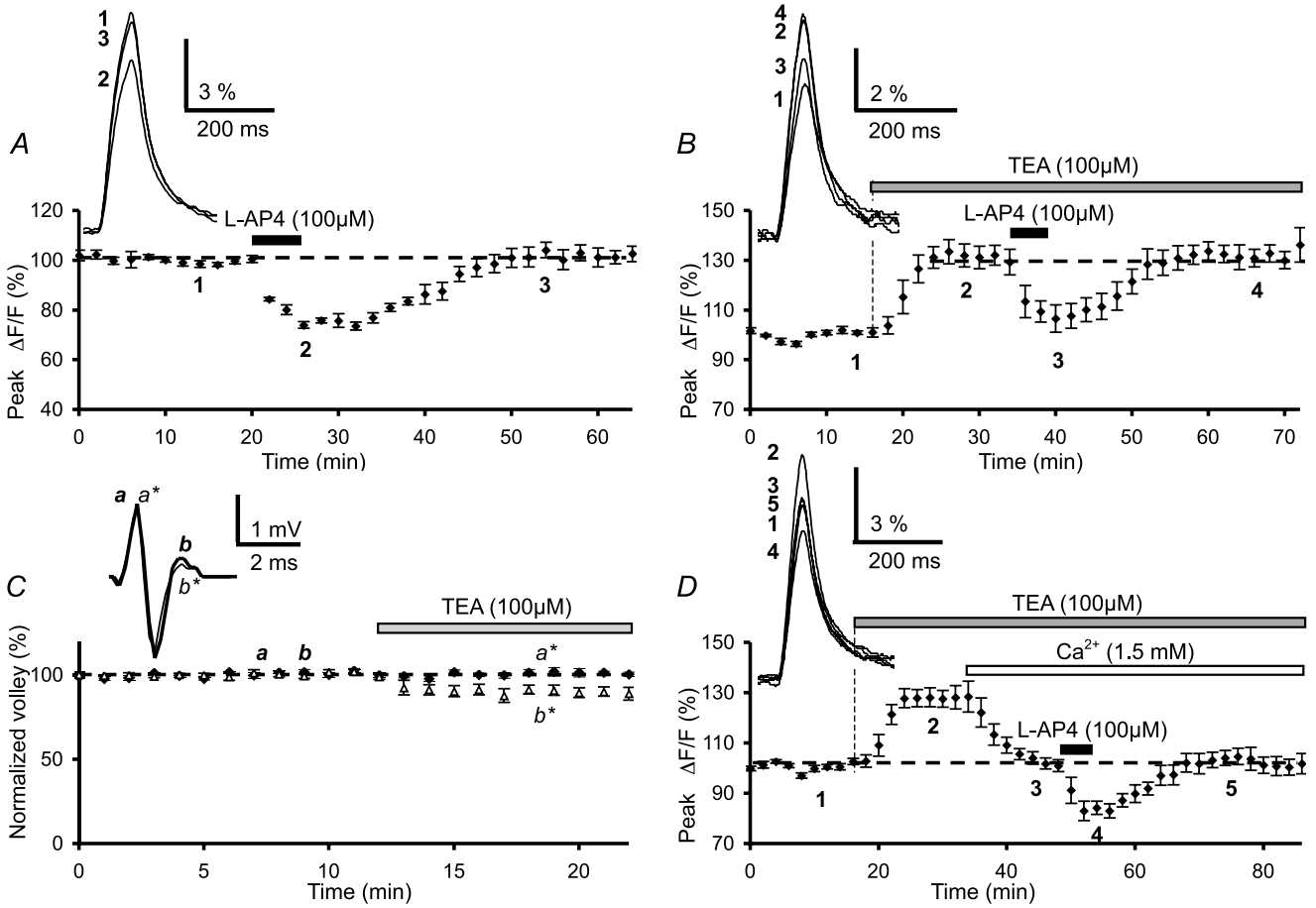


**Figure 1. Lack of effect of the $K^+$ channel blocker, TEA on L-AP4-mediated inhibition of presynaptic $Ca^{2+}$ influx evoked by parallel fibre (PF) stimulation**
*A*, effect of L-AP4 on presynaptic $Ca^{2+}$ influx elicited by PF stimulation. The plot represents the normalized amplitudes of peak Fluo-4FF fluorescence transients ($\Delta F/F$) evoked by 5 PF stimulations (delivered at 100 Hz), plotted as a function of time before (1), during (2) and after (3) bath application of 100 $\mu$M L-AP4 (horizontal filled bar). Each point is the mean ± SEM of 6 separate experiments. The inset on the left displays superimposed averaged fluorescence transients in one of these experiments, recorded at the indicated times. *B*, plot of normalized amplitudes of peak fluorescence transients before, during bath application of TEA (200 $\mu$M) and during co-application of L-AP4 (100 $\mu$M) ($n = 5$). The insets are the same as in *A*. Note that application of TEA in standard extracellular medium (2 mM $CaCl_2$) enhanced the amplitude of the fluorescence transients, but did not prevent their inhibition by L-AP4. *C*, plot of the normalized amplitude of presynaptic PF volleys recorded as field potentials in the molecular layer, against time before and during bath application of TEA (100 $\mu$M) ($n = 4$). The inset on the left shows an example of averaged PF volleys (25–50 consecutive responses) recorded at the indicated times under control conditions (thick trace) and in the presence of TEA (thin trace). Note that TEA altered the PF volleys: while the amplitude of the first volley was unaffected (*a*, control *versus* *a**, under TEA, black diamonds), in contrast the amplitude of the second volley was slightly reduced (*b*, control *versus* *b**, under TEA, open triangles). *D*, same as in *B* after lowering the extracellular $Ca^{2+}$ concentration from 2 to 1.5 mM ($n = 5$). The insets show averaged fluorescence transients in one of these experiments.

with the results published by Daniel & Crepel (2001) and Abitbol *et al.* (2008), 5 min bath application of the broad-spectrum group III mGluR agonist, L-AP4, at a saturating concentration of 100 $\mu$M, reversibly decreased the amplitude of presynaptic $Ca^{2+}$ influxes evoked by PF stimulations by 25.3 ± 2.3% ($n = 6$, Fig. 1*A*).

### TEA-sensitive, G protein-gated inwardly rectifying (Tertiapin Q-sensitive) or two-pore-domain presynaptic $K^+$ channels do not mediate the depressant effect of mGluR4 activation on presynaptic $Ca^{2+}$ influx

Axonal voltage-dependent $K^+$ channels play a major role in fibre excitability and as such influence the presynaptic waveform, $Ca^{2+}$ entry into presynaptic terminals and ultimately neurotransmitter release in mammalian central nervous system synapses (Sabatini & Regehr, 1997). In our previous study (Daniel & Crepel, 2001) we found that application of 1 mM 4-AP, a voltage-sensitive $K^+$ channel blocker, abolished the depressant effect of L-AP4 on PF presynaptic $Ca^{2+}$ influxes. Since at this concentration 4-AP (i) is fairly unselective (Coetzee *et al.* 1999) and (ii) strongly alters PF volleys by reducing the positive-going phase of the waveform indicating a slowing of spike repolarisation (Brown *et al.* 2004), we chose to renew these experiments employing this blocker at a lower concentration (200 $\mu$M 4-AP). As described by Brown *et al.* (2004) for 1 mM 4-AP, we found that bath application of 200 $\mu$M 4-AP consistently altered PF volleys by reducing the positive-going phase of the waveform ($n = 4$, Supplementary Fig. S1*A*). In addition, bath application of 200 $\mu$M 4-AP increased the duration (185% ± 10%) and the amplitude (434% ± 105%) of the presynaptic fluorescence transients ($n = 10$, Supplementary Fig. S1*B*) compared to those recorded in control conditions. As such we abandoned 4-AP and used another $K^+$ channel blocker, tetraethyl ammonium (TEA). In agreement with Brown *et al.* (2004), bath application of TEA (100 $\mu$M) was not entirely without effect on PF volleys ($n = 4$, Fig. 1*C*), or $Ca^{2+}$ transient amplitudes that increased to 131.5 ± 4.5% of control ($n = 5$, Fig. 1*B*). Despite the fact that TEA affected presynaptic volleys and $Ca^{2+}$ influxes, subsequent bath application of L-AP4 (100 $\mu$M) reduced the amplitude of presynaptic $Ca^{2+}$ transients evoked by PF stimulations (Fig. 1*B*). Indeed, from this TEA-induced plateau (representing increased $Ca^{2+}$ transients after a 15 min application of 100 $\mu$M TEA), co-application of L-AP4 (100 $\mu$M) depressed these transients by 19.8 ± 2.3% ($n = 5$). This value is not significantly different ($P > 0.1$) to that recorded in control experiments.

In order to evaluate the effect of mGluR4 activation on evoked $Ca^{2+}$ transients with amplitudes comparable to those recorded in control saline, we reduced the concentration of extracellular $Ca^{2+}$ from 2 to 1.5 mM (osmolarity was maintained by adjusting the extracellular $Mg^{2+}$ concentration) (Fig. 1*D*). Under these conditions, L-AP4 (100 $\mu$M) depressed $Ca^{2+}$ transients by 20.5 ± 1.8% ($n = 5$), a value not significantly different to that obtained under control conditions ($P > 0.1$). However, even if the singular effect of TEA alone on evoked presynaptic volleys and presynaptic $Ca^{2+}$ influxes warrants careful interpretation, these data show that L-AP4-induced depressant effects on PF presynaptic $Ca^2$ influx are independent of TEA-sensitive $K^+$ channels.

We then hypothesized that other $K^+$ channels could be implicated in this mGluR4-mediated effect. Given the importance of two-pore-domain potassium channels (K2P) in the regulation of membrane potential and neuronal excitability, we investigated whether L-AP4 could exert its effects through activating certain K2Ps that are found on cerebellar granule cells like TREK-1 (Talley *et al.* 2001), TASK-1 (Aller *et al.* 2005) and TASK-3 (Watkins & Mathie, 1996). To study these TREK channels in the mGluR4-mediated inhibition of evoked presynaptic $Ca^{2+}$ transients, we determined whether this inhibition was affected by the serotonin re-uptake inhibitor, fluoxetine, a compound that also seems to act as an allosteric blocker of these channels (Kennard *et al.* 2005; Honoré, 2007). We first determined the effect of fluoxetine on the PF volley. As shown in Fig. 2*Aa*, 20 min bath applications of 50 $\mu$M fluoxetine increased the amplitude of the depolarizing wave of the PF afferent volley, indicating an increased granule cell axon excitability ($n = 4$). However, using fluorometric measurements, we observed that similar applications of fluoxetine reduced the amplitude of presynaptic $Ca^{2+}$ transients evoked by PF stimulations by 24.4 ± 7.3% ($n = 7$, Fig. 2*Ab*). From this plateau (representing reduced evoked calcium transients after a 20 min application of 50 $\mu$M fluoxetine), subsequent co-application of L-AP4 (100 $\mu$M) further depressed these calcium transients by 26.1 ± 5.1%, a value not significantly different ($P > 0.8$) to L-AP4-evoked depression in control experiments.

To investigate whether L-AP4 acts by modulating certain TASK channels, we used ruthenium red, a polyvalent cation that has been shown to reduce the open probability of these channels (Musset *et al.* 2006). A 10 min bath application of 10 $\mu$M ruthenium red had no effect on either the PF volley ($n = 4$, Supplementary Fig. S2*A*) or the amplitude or duration of evoked presynaptic $Ca^{2+}$ transients ($n = 10$, Fig. 2*B*). When the 10 min application of ruthenium red (10 $\mu$M) was followed by 5 min co-application of L-AP4 (100 $\mu$M), evoked presynaptic $Ca^{2+}$ transients were depressed by 25.4 ± 2.7%, a value not significantly different ($P > 0.9$) to L-AP4-evoked depression in control experiments ($n = 4$, diamonds, Fig. 2*B*). Since ruthenium red is also

known to block $Ca^{2+}$-sensitive ryanodine receptors, the activation of which result in the liberation of $Ca^{2+}$ from intracellular stores (Bezprozvanny *et al.* 1991), we re-examined the effect of ruthenium red on L-AP4-induced reductions in $Ca^{2+}$ transients after pre-incubation of slices (40 min) in 10 $\mu$M thapsigargin, a molecule that empties intracellular $Ca^{2+}$ stores (Thastrup *et al.* 1990). In these slices, after 10 min application of ruthenium red (10 $\mu$M), co-application of L-AP4 (5 min, 100 $\mu$M) evoked presynaptic $Ca^{2+}$ transients that were depressed by 25.2 ± 4.5% ($n = 7$, circles, Fig. 2*B*), a value not significantly different ($P > 0.9$) to L-AP4 depression in control experiments. These data suggest that the inhibitory action of L-AP4 on presynaptic $Ca^{2+}$ transients cannot be attributed to the activation TREK or TASK channels that are sensitive to fluroxetine and ruthenium red, respectively.

Since certain mGluRs are known to couple to G protein-gated inwardly rectifying $K^+$ channels (GIRKs) (see Niswender *et al.* 2008), we asked whether the mGluR4-mediated inhibition of evoked presynaptic $Ca^{2+}$ influxes involves GIRK activation. We used Tertiapin Q, which selectively inhibits some subtypes of this $K^+$ channel family with nanomolar affinity (Jin & Lu, 1998). We first

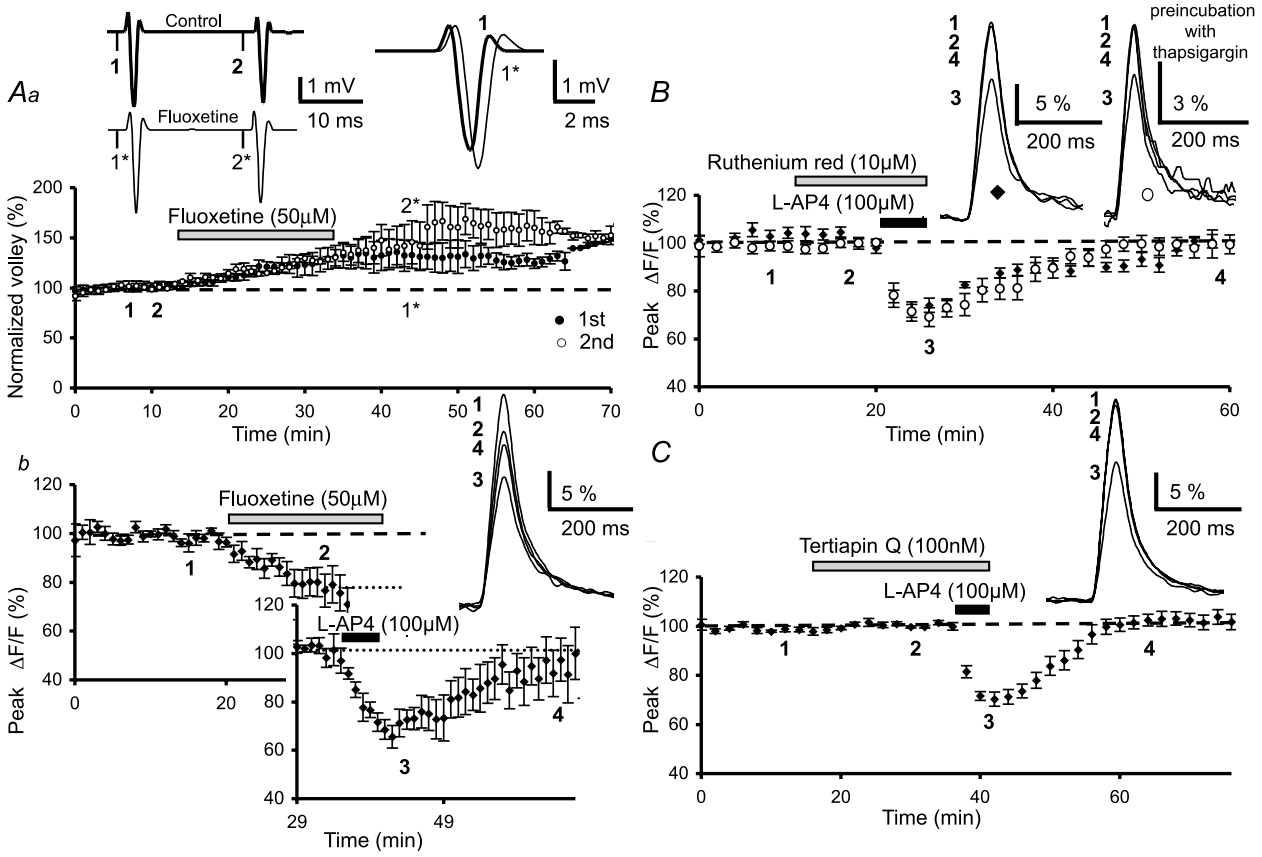


**Figure 2. Lack of effect of the $K^+$ channel blockers fluoxetine, ruthenium red and Tertiapin Q on L-AP4-mediated inhibition of presynaptic $Ca^{2+}$ influx**
*Aa*, plot of the normalized amplitude of PF volleys against time before, during and after bath application of fluoxetine (50 $\mu$M) ($n = 4$). The inset on the left shows examples of averaged PF olleys evoked by two stimuli separated by 20 ms and recorded at the indicated times in control conditions (top, thick trace) and in the presence of fluoxetine (bottom, thin trace). Note that fluoxetine increases the amplitude of the first (black circles) and the second (open circles) PF volley. In addition, note the change in the kinetics of the first PF volley (inset on the right). *Ab*, plot of normalized amplitudes of peak Fluo4-FF fluorescence transients ($\Delta F/F$) recorded as a function of time before, during and after bath application of fluoxetine (50 $\mu$M) and co-application of L-AP4 (100 $\mu$M) ($n = 7$). The top inset displays superimposed averaged fluorescence transients in one of these experiments, recorded at the indicated times. The bottom inset (graph) shows the L-AP4-mediated inhibition of the fluorescence transients normalized to the plateau level obtained after application of fluoxetine. Note that application of fluoxetine alone reduced the amplitude of these fluorescence transients. *B*, the same as in *Ab* before, during and after bath application of ruthenium red (10 $\mu$M) and co-application of L-AP4 (100 $\mu$M) in control slices ($n = 10$, black diamonds) and in slices pre-incubated with thapsigargin (10 $\mu$M) ($n = 7$, open circles). The insets are the same as in *Ab* recorded in control slices (left) and pre-incubated slices (right). *C*, the same as in *Ab* before, during and after bath application of Tertiapin Q (100 nM) and co-application of L-AP4 (100 $\mu$M) ($n = 10$). Top inset as in *Ab*.

determined whether this inhibitor altered the presynaptic waveform. Bath application (20 min) of Tertiapin Q (100 nM) had no effect on the shape of the PF volley ($n = 4$, Supplementary Fig. S2*B*), or the amplitude or the duration of presynaptic fluorescence transients evoked by PF stimulations ($n = 10$, Fig. 2*C*). When the 20 min application of Tertiapin Q (100 nM) was followed by 5 min co-application of L-AP4 (100 $\mu$M), evoked presynaptic $Ca^{2+}$ transients were depressed by $28.9 \pm 2.5\%$ ($n = 10$, Fig. 2*C*), a value not significantly different ($P > 0.3$) to L-AP4-evoked depression in control experiments. Taken together, these data show that the inhibitory action of L-AP4 on presynaptic $Ca^{2+}$ transients cannot be attributed to the activation of Tertiapin Q-sensitive $K^+$ channels.

## mGluR4 activation modulates multiple types of voltage-gated $Ca^{2+}$ channels

There are at least three pharmacologically distinguishable types of VGCCs that synergistically contribute to neurotransmitter release at PF–PC synapses: the $\omega$-agatoxin TK-sensitive P/Q-type, the $\omega$-conotoxin GVIA-sensitive N-type and the SNX-482-sensitive R-type (Mintz *et al.* 1995; Brown *et al.* 2004; Daniel *et al.* 2004). To test the hypothesis that activation of mGluR4 at these synapses reduces glutamate release by selectively inhibiting one or more of these presynaptic VGCCs, we used specific toxins that target these channels.

First, we blocked P/Q-type $Ca^{2+}$ channels with $\omega$-agatoxin TK (250 nM) and investigated the effect of L-AP4 on the remaining fraction of presynaptic $Ca^{2+}$-sensitive fluorescence transients elicited by PF stimulation. Bath application of $\omega$-agatoxin TK (250 nM for 30 min) alone reduced presynaptic $Ca^{2+}$ transients by $33.6 \pm 6.2\%$ ($n = 6$, Fig. 3*A*). From this plateau (representing the reduced $Ca^{2+}$ transients in the presence of $\omega$-agatoxin TK), subsequent application of L-AP4 (100 $\mu$M) further decreased presynaptic $Ca^{2+}$ transients by $25.9 \pm 1.5\%$ ($n = 6$, Fig. 3*A*). The L-AP4 depression was statistically indistinguishable from that observed with bath application of L-AP4 in control conditions ($P > 0.8$). We next investigated the L-AP4-mediated inhibition of presynaptic $Ca^{2+}$ transients after N-type $Ca^{2+}$ channels were blocked by a 30 min bath application of 250 nM $\omega$-conotoxin GVIA. $\omega$-conotoxin GVIA alone reduced presynaptic fluorescence transients by $36.9 \pm 6\%$ ($n = 5$, Fig. 3*B*). From this plateau (representing the reduced $Ca^{2+}$ transients in the presence of $\omega$-conotoxin GVIA), subsequent application of L-AP4 (100 $\mu$M) further decreased presynaptic $Ca^{2+}$ transients by $27.6 \pm 4.4\%$ ($n = 5$, Fig. 3*B*), a value not significantly different ($P > 0.8$) to that observed with L-AP4 application alone. We next blocked R-type $Ca^{2+}$ channels with 30 min bath application of 100 nM SNX-482 and examined the effect of L-AP4 on the remaining fraction of presynaptic $Ca^{2+}$ transients. SNX-482 alone reduced presynaptic fluorescence transients (plateau) by $21.3 \pm 3.5\%$ ($n = 6$, Fig. 3*C*). Subsequent application of L-AP4 (100 $\mu$M) further decreased these transients by $28.9 \pm 4\%$ ($n = 6$, Fig. 3*C*), a value not significantly different to that observed for L-AP4 in control conditions ($P > 0.6$).

Finally, to investigate possible cross-interactions between mGluR4 and the different presynaptic VGCCs, we applied $\omega$-agatoxin TK (250 nM), $\omega$-conotoxin GVIA (250 nM) and SNX-482 (100 nM) simultaneously for 30 min. While bath application of cadmium (100 $\mu$M) totally abolished $Ca^{2+}$-sensitive fluorescence transients elicited by PF stimulation ($n = 4$, Supplementary Fig. S3), the cocktail of $Ca^{2+}$-blocking toxins decreased fluorescence transients by $60.8 \pm 4.7\%$ ($n = 4$, Fig. 3*D*). In the presence of this cocktail, subsequent application of L-AP4 (100 $\mu$M) further reduced these transients, giving an additional reduction in transient amplitude expressed as a percentage of the transients recorded at the plateau level of $28.1 \pm 3.5\%$ ($n = 4$, Fig. 3*D*). This value was not significantly different to that observed in control conditions ($P > 0.4$), or after application of $\omega$-agatoxin TK ($P > 0.5$), $\omega$-conotoxin GVIA ($P > 0.9$) or SNX-482 ($P > 0.8$) alone (see above).

Taken together, these results show that mGluR4 activation does not selectively inhibit P/Q-, N- or SNX-482-sensitive R-type presynaptic VGCCs. Indeed, when a single type of VGCC is selectively blocked, the presynaptic $Ca^{2+}$ influx resulting from activation of other types of calcium channels, whatever they may be, is still inhibited by pharmacological activation of mGluR4, and to the same degree as mGluR4 activation in control saline. mGluR4 activation by L-AP4 appears to modulate all presynaptic terminal VDCCs to the same degree.

## MAPKs or PI3Ks are not implicated in mGluR4-mediated depression of presynaptic $Ca^{2+}$ influx

Previous studies have demonstrated that in cultured cerebellar granule cells, mGluR4s are coupled to MAPKs and PI3Ks and that these pathways are involved in the neuroprotective role of mGluR4s (Iacovelli *et al.* 2002). In order to determine if these pathways are also implicated in mGluR4-mediated depression of PF $Ca^{2+}$ influx, in separate experiments we examined the effect of L-AP4 under conditions in which these kinases were blocked with PD98059 and LY294002, cell-permeable inhibitors of MAPKs and PI3Ks, respectively (Vlahos *et al.* 1994; Alessi *et al.* 1995). A 20 min bath application of either PD98059 (20 $\mu$M) ($n = 8$, Fig. 4*A*) or LY294002 (1 to 50 $\mu$M) ($n = 8$, Fig. 4*B*) had no effect on fluorescence transients evoked by PF stimulation. When PD98059

was followed by 100 $\mu$M L-AP4, $Ca^{2+}$ transients were reduced by 24.9 ± 1.9%, ($n = 8$, Fig. 4*A*), a value not significantly different to that observed with L-AP4 alone ($P > 0.4$). Similarly, L-AP4 application in the presence of LY294002 reduced presynaptic $Ca^{2+}$ influx by 27 ± 1.7% on average ($n = 8$, Fig. 4*B*), again, a value statistically indistinguishable from that observed in control conditions ($P > 0.8$). As PD98059 and LY294002 were prepared in DMSO (final concentration 0.1%), we verified that DMSO had no effect under our experimental conditions. After 3.5 h of slice pre-incubation in DMSO (final concentration 0.3%), L-AP4 still depressed fluorescence transients by 22.1 ± 2.8% ($n = 5$, Fig. 4*C*), a value not significantly different to that observed in control conditions (no pre-incubation, $P > 0.2$). Taken together, these data clearly indicate that the mGluR4-mediated depression of PF $Ca^{2+}$ transients involves neither the MAPK nor the PI3K signalling pathway.

We then verified that in our conditions PD98059 and LY294002 actually inhibit, respectively, MAPK and PI3K activities. In cerebellar culture, it has been demonstrated that phorbol ester-induced long-term depression (LTD) of synaptic transmission between granule cells and PCs is mediated through activation

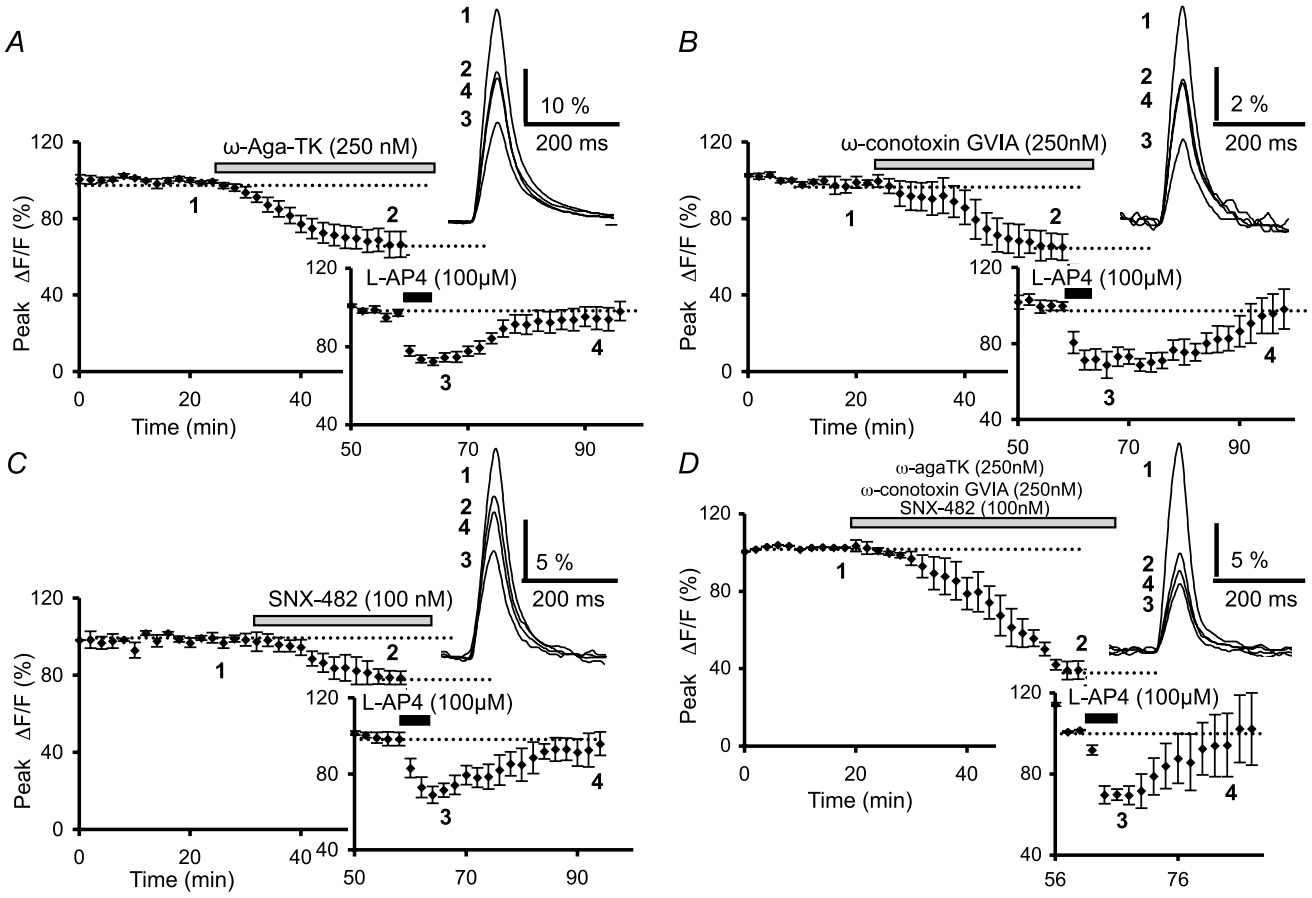


**Figure 3. L-AP4-mediated inhibition of presynaptic $Ca^{2+}$ influx results from modulation of multiple types of VGCCs**

*A*, the plot represents normalized amplitudes of peak fluorescence transients recorded as a function of time before, during and after sequential bath application of $\omega$-agatoxin TK (250 nM) and L-AP4 (100 $\mu$M) ($n = 6$). The top inset displays superimposed averaged fluorescence transients in one of these experiments recorded at the indicated times. The bottom inset (graph) shows the L-AP4-mediated inhibition of the fluorescence transients normalized to the plateau level obtained after application of $\omega$-agatoxin TK. *B*, the same as in *A* with sequential application of $\omega$-conotoxin GVIA (250 nM) and L-AP4 (100 $\mu$M) ($n = 5$). The insets are the same as in *A*. *C*, the same as in *A* with sequential application of SNX-482 (100 nM) and L-AP4 (100 $\mu$M) ($n = 6$). The insets are the same as in *A*. *D*, time course of fluorescence transients before, during and after bath application of combined toxin $\omega$-agatoxin TK (250 nM), $\omega$-conotoxin GVIA (250 nM) and SNX-482 (100 nM), followed by application of L-AP4 (100 $\mu$M) ($n = 4$). The insets show averaged fluorescence transients in one of these experiments (top) and the L-AP4-mediated inhibition of these transients normalized to the plateau level obtained after application of combined toxin treatment (bottom).

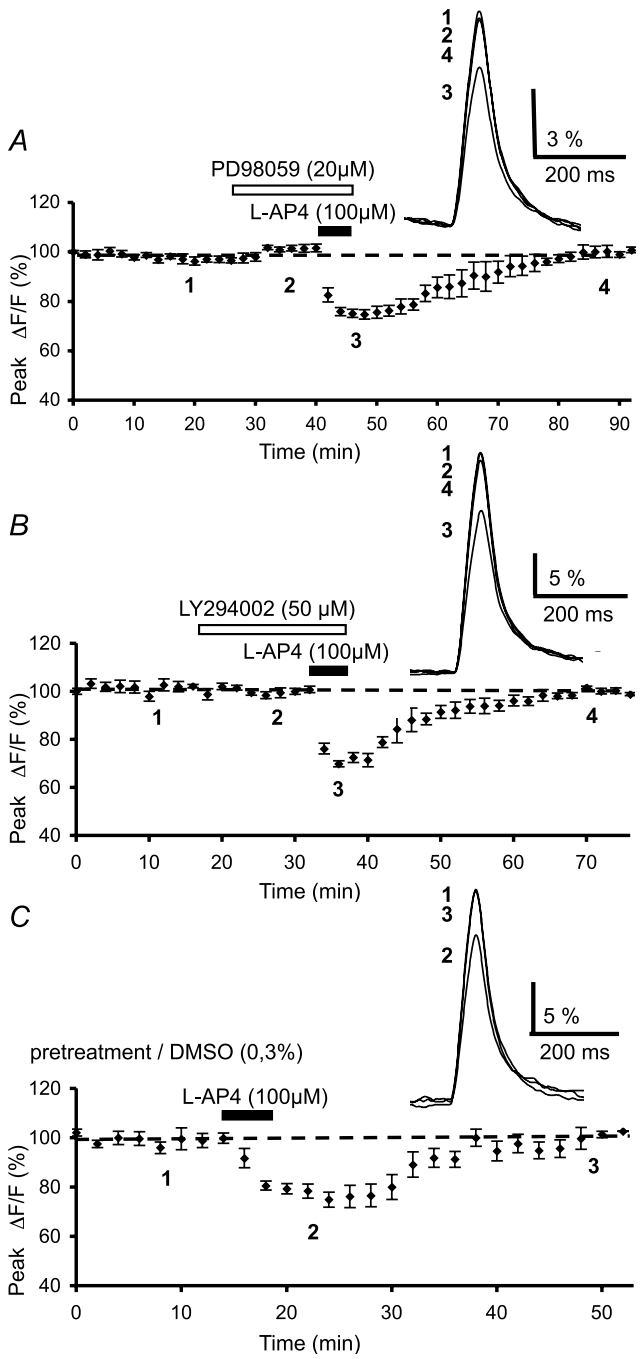


**Figure 4. Lack of effect of MAPK and PI3K blockade on L-AP4-mediated inhibition of presynaptic $Ca^{2+}$ influx**
*A*, plot of normalized amplitudes of peak fluorescence transients before, during and after sequential bath application of the MAPK inhibitor, PD98059 (20 $\mu$M) and L-AP4 (100 $\mu$M) ($n = 8$). The inset represents the superimposed averaged fluorescence transients in one of these experiments. *B*, same as in *A* with sequential bath application of the PI3K inhibitor, LY294002 (50 $\mu$M) and L-AP4 (100 $\mu$M) ($n = 8$). Inset as in *A*. *C*, same as in *A* with bath application of L-AP4 (100 $\mu$M) after pre-incubation of slices in DMSO (0.3%) for 3.5 h ($n = 5$). Insets as in *A*.

of the MAPK pathway, which may either be a downstream target of PKC or act in parallel with PKC, and that PD98059 completely blocked this form of synaptic plasticity (Endo & Launey, 2003). In cerebellar slices, we performed whole-cell patch-clamp recordings to measure PC excitatory postsynaptic currents (EPSCs) evoked by PF stimulation. A 10 min bath application of phorbol esters (PMA, 200 nM) resulted in a long-term decrease in the amplitude of PF-mediated EPSCs ($23.9 \pm 2.8\%$, $n = 4$, Supplementary Fig. S4*A*), as previously demonstrated (Endo & Launey, 2003). After 15 min of pretreatment with PD98059 (20 $\mu$M), PMA was ineffective in inducing LTD ($n = 4$, Supplementary Fig. S4*B*), demonstrating that in our experimental conditions, MAPK activity was effectively inhibited. In the same preparation, we then tested LY294002 since this inhibitor of PI3K has been shown to block the induction of a nitric oxide (NO)-dependent form of plasticity, long-term potentiation (LTP), at PF–PC synapses (Jackson *et al.* 2010). In agreement with these observations, bath application of the NO donor DEA/NO (10 $\mu$M) led to a robust long-term increase in PF-mediated EPSC ($40.5 \pm 4.4\%$ and $59.6 \pm 5.2\%$, respectively, for the second and first response, $n = 4$, Supplementary Fig. S4*C*). As previously demonstrated (Jackson *et al.* 2010), LY294002 (50 $\mu$M) pretreatment for 15 min entirely blocked LTP ($n = 4$, Supplementary Fig. S4*D*), showing that this compound effectively blocks PI3K signalling in our experimental conditions.

## mGluR4-mediated inhibition of presynaptic $Ca^{2+}$ influx does not require the $G_{i/o}$ protein–cAMP–PKA signalling cascade

The classical transduction pathway activated by pharmacological stimulation of mGluR4 is the inhibition of adenylyl cyclase (AC) and protein kinase A (PKA), via $G_{i/o}$ proteins (Prezeau *et al.* 1994). In an attempt to verify that the intracellular signals mediating the L-AP4 inhibition of presynaptic $Ca^{2+}$ influx are part of the $G_{i/o}$ protein–AC cascade in our model, we examined the effect of L-AP4 on presynaptic fluorescence transients after blocking either $G_{i/o}$ proteins, AC or PKA activity. Our results are surprising.

Cerebellar slices were incubated with PTX (2–5 $\mu$g ml$^{-1}$) for 12–19 h before the recording session to inhibit $G_{i/o}$ protein activity. Following PTX pretreatment, the average peak amplitude of the fluorescence transients ($8.4 \pm 1.9\%$ ($\Delta F/F$), $n = 6$) was not significantly different from that recorded in control slices (incubated for the same time in the absence of PTX ($8.6 \pm 2.2\%$ ($\Delta F/F$), $n = 8$) ($P > 0.1$). Bath application of 100 $\mu$M L-AP4 reduced the amplitude of fluorescence transients by $24.1 \pm 2.5\%$ ($n = 9$, Fig. 5*A*), which is not significantly

different to that observed under control conditions ($P > 0.6$). In order to confirm the efficacy of the PTX pretreatment, in a separate series of experiments we activated presynaptic type 1 cannabinoid (CB1) receptors with a specific agonist, WIN55,212-2, after pretreatment of slices in PTX. CB1 receptors are known to activate $G_{i/o}$ proteins and decrease presynaptic $Ca^{2+}$ influx (Daniel *et al.* 2004) and synaptic transmission (Kreitzer & Regehr, 2002) at PF–PC synapses. In PTX-pretreated slices, 30 min bath application of WIN55,212-2 (1 $\mu$M) had no significant effect on evoked PF $Ca^{2+}$ transients ($n = 9$, Fig. 5*B*). This result concurs with that previously shown by Daniel *et al.* (2004), and demonstrates unambiguously that PTX was effective in our experimental conditions. In these PTX-pretreated slices, we applied L-AP4 after WIN55,212-2 treatment and observed a reduction in evoked $Ca^{2+}$ transients of $22.4 \pm 2.9\%$, a value not significantly different from the L-AP4 effect in control conditions ($P > 0.8$) ($n = 9$, Fig. 5*B*). Thus, it appears that the L-AP4-mediated depression of presynaptic $Ca^{2+}$ influx does not depend on the activation of $G_{i/o}$ proteins.

Given our results obtained after $G_{i/o}$ protein inactivation, in separate experiments we tested the involvement of AC in the L-AP4-mediated depression of evoked fluorescence transients. We used two inhibitors of

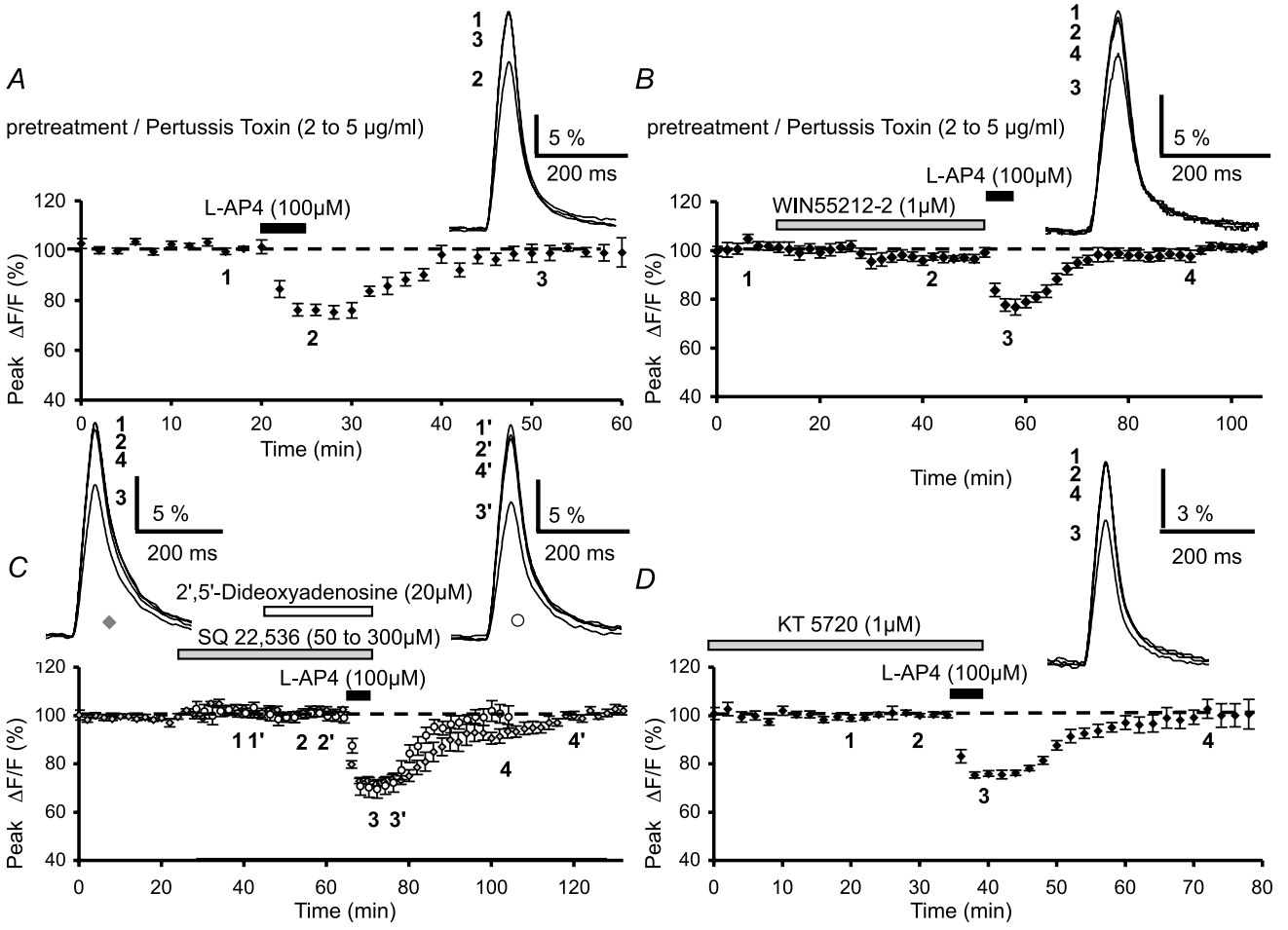


**Figure 5. Lack of effect of pertussis toxin treatment, adenylyl cyclase inhibition or PKA blockade on L-AP4-mediated inhibition of presynaptic $Ca^{2+}$ influx**

*A*, plot of normalized amplitudes of peak fluorescence transients before, during and after bath application of L-AP4 (100 $\mu$M) ($n = 9$). Experiments were performed on slices pre-incubated with pertussis toxin (2 to 5 $\mu$g ml$^{-1}$) for 12–19 h. The inset represents the superimposed averaged fluorescence transients in one of these experiments. *B*, same as in *A* with sequential bath application of WIN55,212-2 (1 $\mu$M) and L-AP4 (100 $\mu$M) ($n = 9$), following similar pre-incubation of slices with pertussis toxin. Inset as in *A*. Note that after this pre-treatment WIN55,212-2, in contrast to L-AP4, does not inhibit fluorescence transients. *C*, same as in *A* with sequential bath application of the adenylyl cyclase inhibitors SQ 22,536 (50 to 300 $\mu$M, diamonds, $n = 11$) or 2′,5′-dideoxyadenosine (20 $\mu$M, circles, $n = 6$), and L-AP4 (100 $\mu$M). Insets as in *A*. *D*, plot of normalized amplitudes of peak fluorescence transients before, during and after bath sequential application of the PKA inhibitor, KT 5720 (1 $\mu$M) and L-AP4 (100 $\mu$M) ($n = 9$). Inset as in *A*.

adenylyl cyclase, namely SQ22,536 (Harris *et al.* 1979) and 2′,5′-dideoxyadenosine (Gille *et al.* 2004), both of which are membrane-permeable broad-spectrum inhibitors of AC. Bath application of SQ22,536 (50 to 300 $\mu$M) for 40 min had no effect on fluorescence transients evoked by PF stimulation ($n = 11$, diamonds, Fig. 5*C*). Moreover, this blocker did not prevent the subsequent inhibitory effect of L-AP4, since in the presence of L-AP4, the amplitude of fluorescence transients was reduced by $27.6 \pm 1.5\%$ ($n = 11$, Fig. 5*C*), a value not significantly different to the L-AP4-mediated inhibition in control conditions ($P > 0.7$). Similarly, 20 min bath application of 2′,5′-dideoxyadenosine (20 $\mu$M) had no effect on fluorescence transients evoked by PF stimulation ($n = 6$, circles, Fig. 5*C*). Here again, subsequent application of L-AP4 reduced evoked $Ca^{2+}$ transients by $29.7 \pm 3.8\%$ ($n = 6$, Fig. 5*C*), a value statistically indistinguishable to that observed in control conditions ($P > 0.7$).

As for the PTX experiments described above, we verified that in our conditions SQ22,536 and 2′,5′-dideoxyadenosine inhibit adenylyl cyclase activity. At PF–PC synapses, elevation of cAMP levels by forskolin, an activator of adenylyl cyclase, has been shown to enduringly enhance neurotransmitter release (Salin *et al.* 1996; Chen & Regher, 1997) through a presynaptic mechanism that does not alter resting $Ca^{2+}$ levels or presynaptic $Ca^{2+}$ influx, but rather directly increases the probability of vesicular release (Chen & Regher, 1997). Thus, we reasoned that this long-term enhancement of synaptic strength downstream from $Ca^{2+}$ influx, might provide a selective means to test the efficiency of adenylyl cyclase inhibitors. Again, whole-cell patch-clamp recordings of PCs were performed to record PF-mediated EPSCs. A 10 min bath application of 50 $\mu$M forskolin resulted in a large increase in the amplitude of PF-mediated EPSCs ($168.2 \pm 6.9\%$, $n = 8$, Supplementary Fig. S5*A*). These data are consistent with previous studies (Salin *et al.* 1996; Chen & Regher, 1997, Daniel *et al.* 2004). After pretreatment with SQ22,536 (50 $\mu$M) for 40 min or 2′,5′-dideoxyadenosine (20 $\mu$M) for 20 min, forskolin was ineffective ($n = 4$, Supplementary Fig. S5*B* and *C*), confirming that in our experimental conditions, adenylyl cyclase activity was effectively inhibited. Collectively, these data demonstrate that the mGluR4-mediated depression of presynaptic $Ca^{2+}$ influx does not involve AC.

As a final step in exploring this signalling pathway, we looked for a role of PKA in the L-AP4-mediated inhibition of presynaptic $Ca^{2+}$ influx by examining the L-AP4-mediated reduction in $Ca^{2+}$ transients after inhibition of PKA activity. We employed KT 5720, a specific membrane-permeable inhibitor of PKA that acts with nanomolar affinity (Kase *et al.* 1987). Bath application of 1 $\mu$M KT 5720 for 10 min to 2 h had no effect on evoked fluorescence transients ($n = 9$, Fig. 5*D*), nor did this blocker prevent the inhibitory effect of subsequently applied L-AP4. In the presence of KT 5720, L-AP4 depressed PF fluorescence transients by $24.1 \pm 1.3\%$ on average ($n = 9$, Fig. 5*D*), a value not significantly different to that observed in control conditions ($P > 0.9$). These data demonstrate that the depressant effect of L-AP4 on presynaptic $Ca^{2+}$ influx does not require the modulation of PKA activity.

Taken together, our results show that under our experimental conditions, mGluR4-mediated depression of evoked PF $Ca^{2+}$ influx is independent of the classical $G_{i/o}$ protein–cAMP–PKA transduction pathway.

## mGluR4-mediated inhibition of presynaptic $Ca^{2+}$ influx probably involves the PLC–PKC signalling cascade

As demonstrated by Perroy *et al.* (2000) in cultured cerebellar granule cells, mGluR7s, which are part of the group III family, are functionally coupled to a PLC–PKC signalling pathway. In light of our results showing that L-AP4 does not activate the $G_{i/o}$ protein–AC–PKA transduction pathway, we examined the possibility that PLC and PKC are also intracellular actors in the mechanisms underlying the inhibitory effect of mGluR4 activation on presynaptic $Ca^{2+}$ influx. We began by blocking the PLC pathway by pre-incubating cerebellar slices for 3.5 h in the membrane-permeable PLC inhibitor, U-73122 (10 $\mu$M) (Netzeband *et al.* 1997). We first tested for putative effects of this compound on fluorescence transients evoked by PF stimulation. U-73122 (10 $\mu$M) alone had no effect on these transients ($n = 3$, not illustrated). Co-application of L-AP4 and U-73122 produced a much smaller depression in evoked $Ca^{2+}$ influx ($9.4 \pm 4.2\%$) than that observed with L-AP4 alone ($n = 6$, Fig. 6*A*). This difference was statistically significant ($P < 0.001$). Furthermore, as expected, pre-incubation of slices for 3.5 h with the inactive analogue U-73343 (10 $\mu$M), had no effect *per se* on fluorescence transients ($n = 3$, not illustrated), or on the L-AP4-mediated depression of fluorescence transients ($22.1 \pm 2.5\%$, $n = 5$, Fig. 6*B*), a value not significantly different to that observed in control experiments ($P > 0.3$).

Finally, to ascertain the efficacy of the PLC inhibitor, U-73122 (10 $\mu$M), we analysed the effects of this compound on mGluR1-mediated depression of evoked EPSCs, which in cultured Purkinje cell neurons is known to be due, at least in part, to PLC activation (Netzeband *et al.* 1997). In control experiments, 5 min bath application of DHPG (100 $\mu$M), a selective mGluR1 agonist, induced a transient decrease in the amplitude of PF-mediated EPSCs of $31.2 \pm 4.9\%$ ($n = 4$, Fig. 6*C*), an effect consistent with our previous study (see Fig. 2*B* in Levenes *et al.* 2001). This DHPG-mediated inhibitory effect was attenuated by pre-incubation of slices for

3.5 h with U-73122 (10 $\mu$M), since the magnitude of the transient decrease in PF-mediated responses was only $4.1 \pm 3.1\%$ ($n = 5$, Fig. 6*D*), a value significantly smaller than that observed in control experiments ($P < 0.001$). This finding unambiguously demonstrates that in our experimental conditions, U-73122 (10 $\mu$M) blocked PLC activity.

Furthermore, to illustrate the physiological relevance of a PLC-dependent pathway in the L-AP4-mediated modulation of synaptic transmission at PF–PC synapses, we performed a series of electrophysiological experiments to evaluate whether the L-AP4-induced depression of EPSCs and/or the paired-pulse facilitation (PPF) was impaired after inhibiting PLC at these synapses. PPF has been successfully used at PF–PC synapses as an index of a presynaptic site of action of the group III mGluR broad-spectrum agonist L-AP4 (Pekhletski *et al.* 1996; Miniaci *et al.* 2001; Lorez *et al.* 2003). However, taking into account the fact that U-73122 affects both pre- and postsynaptic compartments our results require careful interpretation. In control experiments, 5 min bath application of 100 $\mu$M L-AP4 reversibly depressed the amplitude of both the first and the second PF-evoked EPSC elicited by two successive stimuli in PCs recorded in voltage-clamp mode. At its peak, the mean decrease in PF-evoked EPSC amplitude was $74.3 \pm 3.5\%$ for the

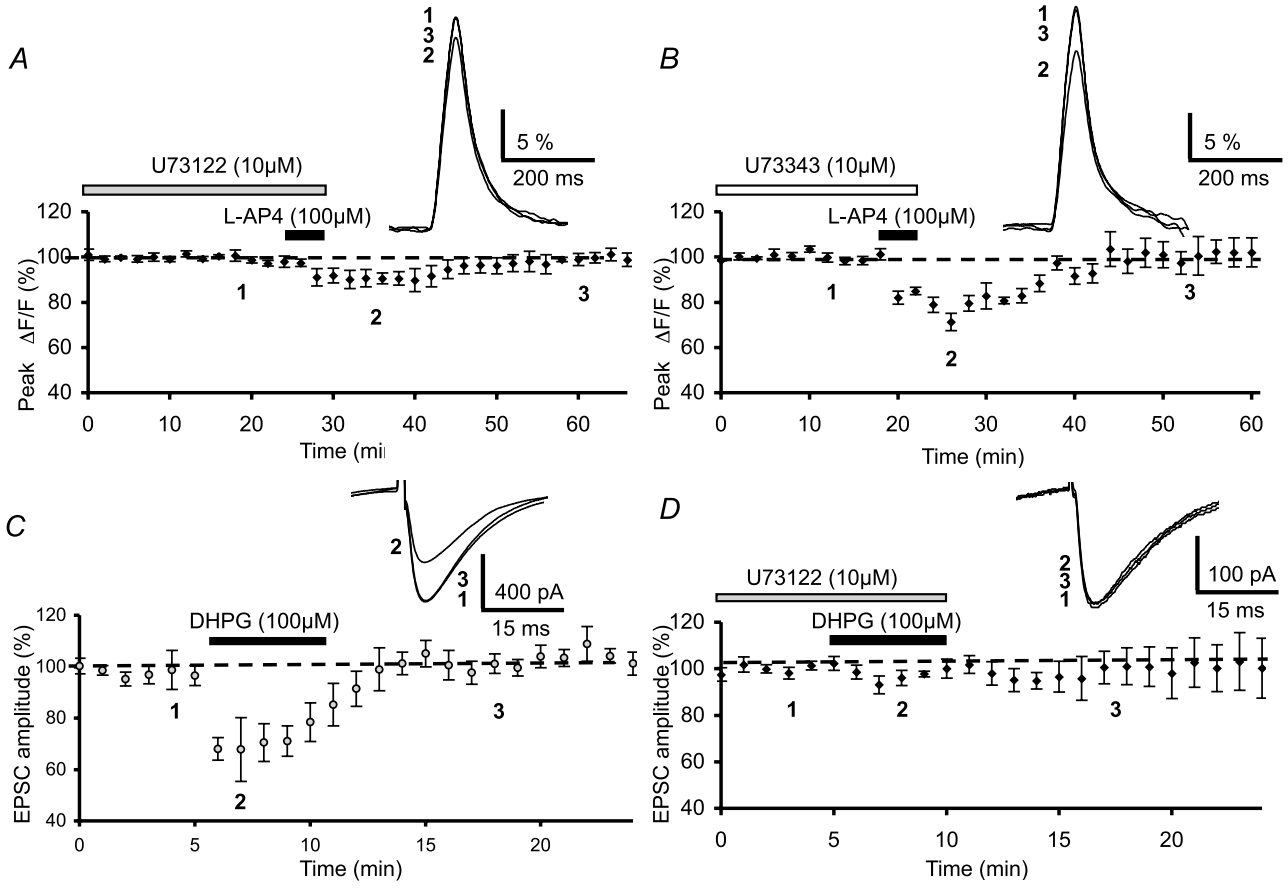


**Figure 6. Effect of phospholipase C blockade on L-AP4-mediated inhibition of presynaptic $Ca^{2+}$ influx and on DHPG-mediated depression of PF synaptic responses**
*A*, plot of normalized amplitudes of peak fluorescence transients before, during and after bath application of L-AP4 (100 $\mu$M) in the presence of U-73122 (10 $\mu$M) ($n = 6$). Note that experiments were performed on slices pre-incubated with the PLC inhibitor, U-73122 (10 $\mu$M) for 3.5 h. The inset represents superimposed averaged fluorescence transients in one of these experiments. *B*, same as in *A* with bath application of L-AP4 (100 $\mu$M) in presence of, and after 3.5 h pre-incubation of slices with the inactive analogue of U-73343 (10 $\mu$M) ($n = 5$). Inset as in *A*. *C*, plot of normalized amplitudes of PF-mediated excitatory postsynaptic currents (EPSCs) as a function of time before, during and after bath application of 100 $\mu$M DHPG in control conditions (circles, $n = 4$). Inset displays superimposed PF-mediated EPSCs recorded in one of each series of experiments recorded at the indicated time. *D*, time course of EPSC amplitude before, during and after bath application of 100 $\mu$M DHPG and example traces (inset), with slices pre-incubated in U-73122 (10 $\mu$M) for 3.5 h (diamonds, $n = 4$). Note that this pre-incubation prevents the DHPG-mediated depressant effect on PF-mediated EPSC amplitude.

first response (grey triangle) and 65.6 ± 2.8% for the second (grey circle) ($n = 10$, Fig. 7*A* top). L-AP4-induced decreases in amplitude were accompanied by a highly significant ($P < 0.01$) increase in mean PPF, from 2.3 ± 0.1 during the control period to 3.5 ± 0.4 at the peak of L-AP4 depressant effect (Fig. 7*A* bottom trace, $n = 10$).

In marked contrast, for the 10 cells tested after pre-treatment with U-73122 (10 $\mu$M), the depressant effect on PF-EPSC amplitude following 5 min bath application of 100 $\mu$M L-AP4 was 23.8 ± 3.7% and 14.4 ± 3% for the first (black circle) and the second (black triangle) EPSCs, respectively ($n = 10$, Fig. 7*B* top). These L-AP4-induced depressions were significantly smaller than those recorded in control experiments for both the first and the second responses ($P < 0.0001$). PPF remained unchanged ($n = 10$, Fig. 7*B* bottom trace, filled squares). In contrast, pretreatment with U-73343 (10 $\mu$M), an inactive analogue

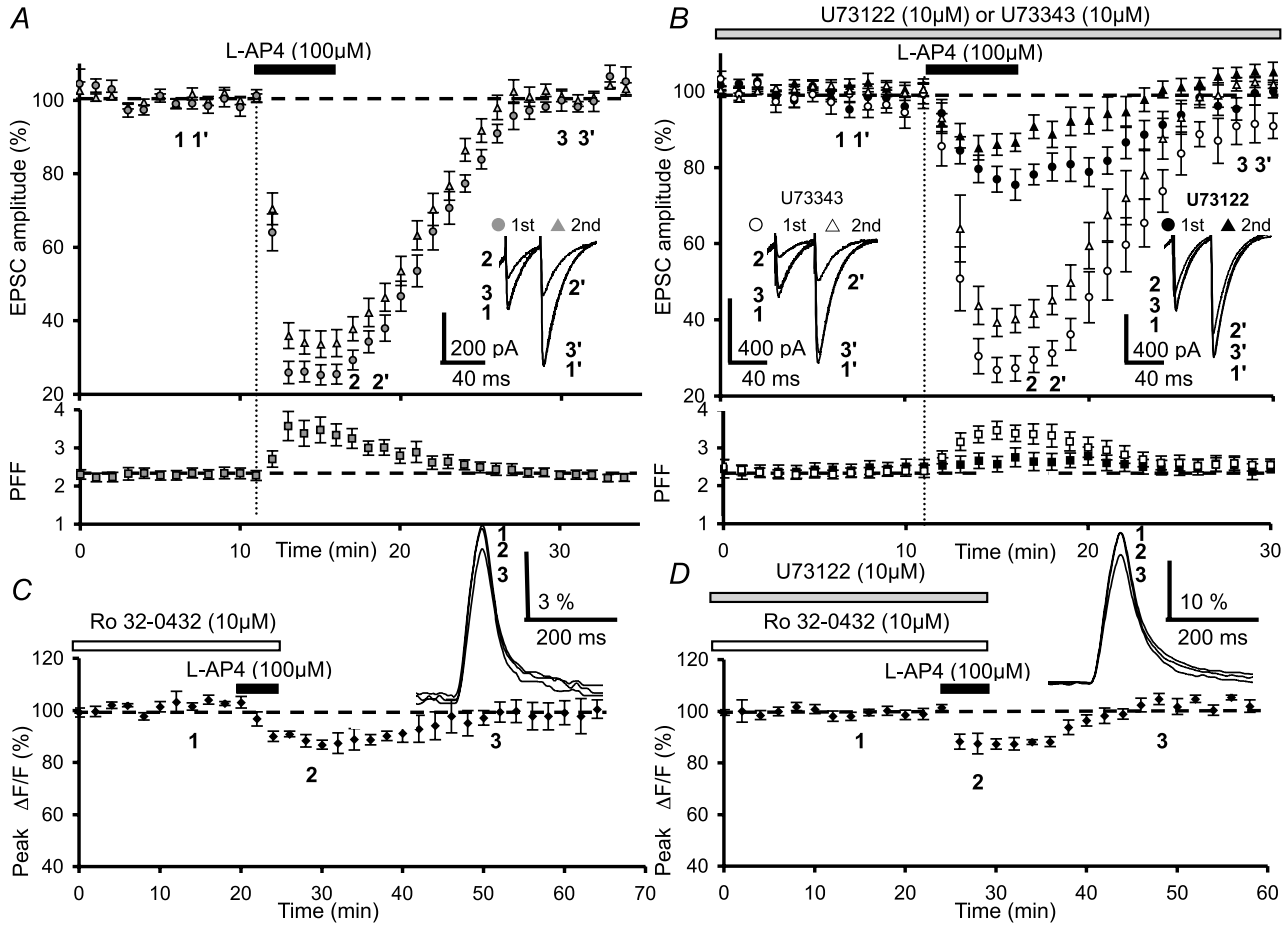


**Figure 7. L-AP4-mediated inhibition of synaptic transmission and presynaptic $Ca^{2+}$ influx involve activation of a PLC–PKC pathway**

*A*, time course of normalized amplitudes of PF-mediated EPSCs before, during and after bath application of 100 $\mu$M L-AP4 ($n = 10$). The inset displays superimposed sweeps of representative PF-evoked EPSCs elicited in one Purkinje cell by 2 successive PF stimulations with an inter-stimulus interval of 40 ms, before (1, 1′), during (2, 2′) and after (3, 3′) agonist application. Each trace is an average of 5–15 consecutive trials. The reversible L-AP4 depression of PF-mediated EPSCs is accompanied by a transient increase in paired-pulse facilitation (PPF). *B*, same as in *A*, but after pre-incubating slices for 3.5 h in the membrane-permeable PLC inhibitor U-73122 (10 $\mu$M, $n = 10$ black symbols), or its inactive analogue U-73343 (10 $\mu$M, $n = 10$, white symbols). Insets as in *A*. Note that pre-incubation with the PLC inhibitor (U-73122, 10 $\mu$M) partially prevents the L-AP4-mediated depressant effect on PF-evoked EPSCs amplitude and the transient increase in PPF, which is not observed with its inactive analogue. *C*, plot of normalized amplitudes of peak fluorescence transients before, during and after bath application of L-AP4 (100 $\mu$M) in the presence of Ro 32-0432 (10 $\mu$M) ($n = 6$). Experiments were performed on slices pre-incubated with Ro 32-0432 (10 $\mu$M) for 1.5 h. The inset represents superimposed averaged fluorescence changes in one of these experiments. *D*, same as in *C* with bath application of L-AP4 (100 $\mu$M) in presence of Ro 32-0432 (10 $\mu$M) and U-73122 (10 $\mu$M) ($n = 7$). Sample traces are shown in the inset. Experiments were performed with slices pre-incubated in Ro 32-0432 and U-73122, for 1.5 h and 3.5 h, respectively.

of the PLC inhibitor, had no effect on the L-AP4-mediated inhibition of PF-EPSC amplitude (71.5 ± 5.6% for the first responses (white triangle) and 58.8 ± 3.9% for the second responses (white circle) ($n = 10$, Fig. 7*B* top). These values were not significantly different to those observed for the first and the second PF-EPSCs in control experiments ($P > 0.2$). In the presence of U-73122, a highly significant ($P < 0.001$) increase in mean PPF was observed, from 2.2 ± 0.1 during the control period to 3.4 ± 0.2 at the peak depressant effect ($n = 10$, Fig. 7*B* bottom trace). The mean PPF at the peak of the L-AP4 depressant effect was not significantly different from that observed in control conditions ($P > 0.3$).

As a final step we examined the putative role of PKC in the mGluR4-mediated depressant effect of presynaptic $Ca^{2+}$ influx using fluometric mesurements. To this end, we incubated slices for 1.5 h in 10 $\mu$M Ro 32-0432, a cell-permeable inhibitor of PKC (Morreale *et al.* 1997). While Ro 32-0432 had no effect on presynaptic $Ca^{2+}$ influx, subsequent co-application of 100 $\mu$M L-AP4 and Ro 32-0432 decreased fluorescence transients by only 11.3 ± 2.1% ($n = 6$, Fig. 7*C*), a value significantly smaller than that observed with L-AP4 alone ($P < 0.001$).

In light of our results showing a role for PLC and PKC in the L-AP4-mediated decreases in evoked $Ca^{2+}$ influx, we finished our study with a set of occlusion experiments to test whether PLC and PKC could interact in mediating the L-AP4-induced effects. After co-incubation of slices in U-73122 (10 $\mu$M) and Ro 32-0432 (10 $\mu$M) (see above), application of 100 $\mu$M L-AP4 decreased fluorescence transients by 12.6 ± 2.7% ($n = 7$, Fig. 7*D*), a value significantly smaller than that observed with L-AP4 alone ($P < 0.001$) but similar to values observed with either U-73122 alone ($P > 0.5$) or Ro 32-0432 alone ($P > 0.8$). These results suggest that PLC and PKC are both involved in L-AP4-induced responses, and probably use the same signalling pathway, because the L-AP4 decreases in $Ca^{2+}$ influx during blockade of both these enzymes are not additive.

Collectively, these data strongly suggest that the mGluR4-mediated depression of presynaptic $Ca^{2+}$ influx can be attributed to the activation of a PLC–PKC pathway.

## Discussion

In this study we investigated the molecular mechanisms underlying mGluR4-mediated depression of presynaptic $Ca^{2+}$ influx at PF–PC synapses. Our principal finding is that the mGluR4-mediated depression requires neither the 'classical' activation of a PTX-sensitive $G_{i/o}$ protein, nor the AC–PKA signalling pathway. We present evidence that at PF–PC synapses, L-AP4 activation of mGluR4 reduces evoked presynaptic $Ca^{2+}$ influx by way of a non-canonical signalling pathway, notably one involving PLC and the subsequent activation of PKC.

### Presynaptic $K^+$ channels are not involved in mGluR4-induced depression of presynaptic $Ca^{2+}$ entry

We previously showed that 4-AP (1 mM) dramatically affected the properties of evoked $Ca^{2+}$ transients and completely abolished the L-AP4-mediated inhibition of these transients (Daniel & Crepel, 2001). However, it has since been demonstrated that 1 mM 4-AP greatly affects the PF afferent volley and prevents spike repolarisation (Brown *et al.* 2004). As such we re-investigated the mGluR4–$K^+$ channel 4-AP-sensitive link using lower concentrations (200 $\mu$M) of this blocker. Even at this concentration, 4-AP profoundly altered both the presynaptic volley and the presynaptic $Ca^{2+}$ influx, rendering this pharmacological tool inappropriate for studying presynaptic $Ca^{2+}$ events. Thus, in order to investigate the putative role of $K^+$ channels in the L-AP4-mediated depression of presynaptic $Ca^{2+}$ influx, we used TEA, another blocker of $K^+$ channels that has less marked effects on $Ca^{2+}$ influx and only minor effects on the presynaptic waveform (Brown *et al.* 2004). We conclude that presynaptic TEA-sensitive $K^+$ channels are unlikely to contribute to the mGluR4-mediated inhibition of presynaptic $Ca^{2+}$ influx. Why in our previous study (Daniel & Crepel, 2001) 1 mM 4-AP hindered L-AP4-induced decreases in presynaptic $Ca^{2+}$ transient amplitude may lie in the fact that this compound greatly slowed the time course of the $Ca^{2+}$ signals, even in low extracellular $Ca^{2+}$ concentrations. In these conditions a residual increase in presynaptic cytosolic $Ca^{2+}$ levels might contribute to profound alteration of $Ca^{2+}$ signalling in active zones where mGluR4 is present (Mateos *et al.* 1999). Thus, these 4-AP-induced variations in presynaptic $Ca^{2+}$ transients that are not observed either in control conditions or in the presence of low concentrations of TEA, could explain at least in part the puzzling lack of effect of L-AP4 on evoked calcium influx in the presence of 1 mM 4-AP.

Along the same line, we show that Tertiapin Q, at concentrations known to inhibit certain inward rectifying potassium channels including GIRK 1 and 4 (Jin & Lu, 1998), affected neither the shape of the PF volley nor the amplitude or duration of evoked presynaptic $Ca^{2+}$ transients (Daniel & Crepel, 2001). Since Tertiapin Q had no effect on L-AP4 depression of evoked presynaptic $Ca^{2+}$ influx, we conclude that GIRK 1 and 4 do not contribute to the L-AP4-induced depression of PF–PC synaptic transmission.

Recent studies have revealed that two-pore-domain $K^+$ channels (K2P) that contribute to the $K^+$ leak current are widely expressed throughout the central nervous system. TREK-1 (Talley *et al.* 2001), TASK-1 (Aller *et al.* 2005) and TASK-3 (Watkins & Mathie 1996) channels have been localized to cerebellar granule cells. These channels are tightly regulated by numerous G protein-coupled receptors, including mGluR4 (Cain *et al.* 2008), and may

modulate neuronal excitability through their contribution to background membrane currents (Goldstein *et al*. 2001; Bayliss *et al*. 2003; Aller *et al*. 2005). We show that neither TREK nor TASK are responsible for the mGluR4-mediated inhibition of evoked presynaptic $Ca^{2+}$ transients in PFs, since inhibiting TREK-1 channels with fluoxetine, or TASK 1 and TASK-3 channels with ruthenium red, did not affect the L-AP4-induced reduction in these transients. However, these results must be interpreted with caution since neither fluoxetine nor ruthenium red were developed as specific K2P channel blockers but have simply been shown to inhibit channel activity.

### All identified types of $Ca^{2+}$ channels are involved in mGluR4 depression of presynaptic $Ca^{2+}$ entry

Glutamate release at PF–PC synapses is tightly regulated by at least three types of VGCCs that are pharmacologically distinct: the $\omega$-agatoxin TK-sensitive P/Q-type, the $\omega$-conotoxin GVIA-sensitive N-type and the SNX-482-sensitive R-type channels (Mintz *et al*. 1995; Brown *et al*. 2004; Daniel *et al*. 2004). In the CNS, group III mGluR activation depresses transmitter release by directly inhibiting N- or/and P/Q-type VGCCs (Takahashi *et al*. 1996; Millan *et al*. 2002; Capogna, 2004; Rusakov *et al*. 2004; Guo & Ikeda, 2005; Woodhall *et al*. 2007), but not R-type VGCCs (Woodhall *et al*. 2007). We studied the role of these VGCCs in the mGluR4-induced depression of evoked presynaptic $Ca^{2+}$ transients by inhibiting P/Q-, N- and SNX-482-sensitive R-type $Ca^{2+}$ channels individually and then collectively, using pharmacological compounds selective for each type of VGCC. Our results demonstrate that $\omega$-agatoxin TK, $\omega$-conotoxin GVIA and SNX-482 applied separately or together reduced the peak amplitude of evoked presynaptic fluorescence transients but never entirely eliminated them. It must be noted, however, that evoked calcium transients were entirely blocked by application of cadmium. This suggests that either we used non-saturating concentrations of each toxin or that other $Ca^{2+}$ channels, not inhibited by these toxins, are functional on PF terminals. In any case, even if these concentrations were non-saturating, at least a portion of each type of $Ca^{2+}$ channel was inhibited after which L-AP4 reduced remaining presynaptic $Ca^{2+}$ transients to the same extent as under control conditions. Taken together, these results show that mGluR4 activation by L-AP4 does not selectively inhibit any one kind of $Ca^{2+}$ channel. Rather, this receptor appears to modulate all types of $Ca^{2+}$ channel present in the presynaptic terminals to the same degree.

### mGluR4-mediated inhibition of presynaptic $Ca^{2+}$ entry does not involve activation of MAPKs or PI3Ks

There is evidence that group III mGluRs can also activate MAPK and PI3K. In fact, it has been shown that in cultured cerebellar granule cells, mGluR4s are functionally coupled to both MAPKs and PI3Ks (Iacovelli *et al*. 2002). In our study, selective pharmacological blockade of each of these kinases (MAPK or PI3K) showed that neither of these proteins is involved in the L-AP4-induced depression of evoked presynaptic $Ca^{2+}$ influx.

### mGluR4-mediated inhibition of presynaptic $Ca^{2+}$ entry does not require activation of the $G_{i/o}$ protein–AC–PKA cascade

One of the most important finding of our study is that the mGluR4-mediated depression of presynaptic $Ca^{2+}$ transients is not coupled to the classical group III mGluR transduction pathway described in most other systems. Indeed, these receptors are generally linked to $G_{i/o}$ proteins that inhibit AC activity resulting in a decrease in intracellular cAMP levels and PKA activity (Prezeau *et al*. 1994; Neil *et al*. 1996; Conn & Pin, 1997). It must be mentioned, however, that presynaptic group III mGluRs can be positively coupled to AC and subsequently activate PKA (Evans *et al*. 2001). In our study, inactivation of $G_{i/o}$ proteins with PTX had no effect on the inhibition of presynaptic $Ca^{2+}$ transients following mGluR4 activation. Along the same line, pretreatment with the non-specific AC inhibitors SQ22,536 or 2′,5′-dideoxyadenosine, or with the PKA inhibitor KT 5720, had no effect on the reduction of presynaptic $Ca^{2+}$ transients following mGluR4 activation. Taken together these results, albeit surprising, suggest that mGluR4-mediated depression of $Ca^{2+}$ influx at PF terminals does not involve the activation of either $G_{i/o}$ PTX-sensitive proteins, AC or PKA.

### PLC–PKC-dependent signalling pathways are involved in the mGluR4-mediated inhibition of presynaptic $Ca^{2+}$ entry

Finally, we investigated whether a new signalling pathway involving the activation of PLC and PKC could underlie the mGluR4-mediated depression of evoked presynaptic $Ca^{2+}$ influx at PF terminals. We show that selective inhibition of either PLC or PKC significantly reduced the magnitude of the L-AP4-mediated depression of presynaptic $Ca^{2+}$ transients. These findings strongly suggest that mGluR4 depresses presynaptic $Ca^{2+}$ influx by way of a PLC–PKC intracellular signalling pathway. These results are in line with the observation that in cultured cerebellar granular cells, the activation of another type of the group III mGluR family, mGluR7, inhibits P/Q VGCCs by a PLC and PKC-dependent pathway (Perroy *et al*. 2000).

In conclusion, we present evidence for a new signalling pathway for PF mGluR4 receptors that involves PLC and/or PKC-dependent signalling cascades responsible for the reduction of evoked presynaptic $Ca^{2+}$ transients.

However, several important questions are pending. For example, what are the intermediate molecular players between mGluR4 and PLC/PKC (for example, Gq proteins) or between PLC/PKC and $Ca^{2+}$ channels? Does mGluR4 activation also directly affect neurotransmitter exocytosis processes downstream of presynaptic $Ca^{2+}$ influx, as has been shown in cultured cerebellar granule cells (Chavis *et al.* 1998)? A recent study has shown that mGluR4 can sequester Munc18-1, a binding partner of syntaxin-1, which is in turn a pivotal constituent of the SNARE complex crucial for exocytosis of synaptic vesicles (Nakajima *et al*. 2009). Activation of mGluR4 might then reduce vesicular liberation of glutamate through the effects of Munc18-1 sequestration on SNARE activity. It remains to be determined whether an mGluR4 effect on neurotransmitter liberation is direct and/or whether it requires the activation of a specific intracellular signalling pathway, and if so, which pathway is involved.

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## Author contributions

Conception and design of the experiments: K.A. and H.D. Collection, analysis and interpretation of data: K.A., H.McL., T.B. and H.D. Drafting and revising the article for important intellectual content: K.A., H.McL. and H.D. All authors approved the final version.

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