Glutamate as a candidate retrograde messenger at interneurone–Purkinje cell synapses of rat cerebellum

M. Glitsch, I. Llano and A. Marty*

Arbeitsgruppe Zelluläre Neurobiologie, Max-Planck-Institut für biophysikalische Chemie, Göttingen, Germany

1. Depolarization-induced suppression of inhibition (DSI) is a form of synaptic plasticity which involves a retrograde messenger. We have performed experiments in Purkinje cells of rat cerebellar slices to determine the nature of this messenger.

2. DSI is mimicked by 2-(2,3-dicarboxycyclopropyl)-glycine (DCG-IV), a specific agonist of group II metabotropic glutamate receptors (mGluRs).

3. DSI is reduced if transmitter release is inhibited by saturating doses of DCG-IV.

4. Both DSI and DCG-IV-induced inhibition are inhibited by L-2-amino-3-phosphonopropionic acid (L-AP3), a drug which interferes with several subtypes of mGluRs.

5. DSI is reduced if synaptic activity is enhanced by application of forskolin.

6. We propose that glutamate or a glutamate-like substance is the retrograde messenger implicated in DSI, and that the inhibition resulting from presynaptic glutamate binding is mediated by a decrease in the presynaptic concentration of cAMP.

Retrograde messengers have long been suspected to play a role in synaptic integration in the brain but their chemical nature and their mode of action have remained elusive (reviewed in Bliss & Collingridge, 1993; Alger & Pitler, 1995). One clear case for retrograde synaptic signalling is depolarization-induced suppression of inhibition (DSI), a transient decrease of transmitter release observed at GABAergic synapses both in the cerebellum and hippocampus upon postsynaptic depolarization (Llano, Leresche & Marty, 1991; Pitler & Alger, 1992; Vincent, Armstrong & Marty, 1992; Vincent & Marty, 1993; Pitler & Alger, 1994). In the cerebellum DSI has two components, which are both dependent on a postsynaptic rise of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). One component, observable in the presence of tetrodotoxin (TTX), is manifest as a decrease in the rate of miniature IPSCs (mIPSCs). The other component involves a modification in the excitability of the axonal endings, and can be transmitted along the presynaptic axonal arborization to inhibit synapses onto unstimulated postsynaptic cells (Vincent & Marty, 1993). This second component is mainly responsible for the inhibition of spontaneous IPSCs (sIPSCs) seen in the absence of TTX upon postsynaptic depolarization. In the hippocampus, no DSI is observed in the presence of TTX, indicating that the first component is negligible (Alger & Pitler, 1995).

Over the past years, we have screened several potential retrograde messengers for a role in the induction of DSI in the cerebellum. Arachidonic acid, nitric oxide, carbon dioxide, adenosine and GABA, among others, all gave negative results. Here we report results indicating that glutamate, or a glutamate-like substance, could be the messenger.

METHODS

Tight-seal whole-cell recordings were obtained from Purkinje cells as explained previously (e.g. Vincent & Marty, 1993). Briefly, rats aged 11–13 days were decapitated following cervical dislocation, and sagittal cerebellar slices of 200 μm thickness were prepared. The recording chamber was perfused (1·5 ml min^{-1}) with a saline containing (mM): 125 NaCl, 2·5 KCl, 1·25 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$ and 10 glucose, equilibrated with a 95% O_2–5% CO_2 mixture (pH 7·3). For recordings of spontaneous IPSCs (sIPSCs), 1 μM bicuculline methochloride was added to this saline to reduce the amplitude of IPSCs and to minimize the probability that large IPSCs would trigger dendritic action potentials. For recordings of mIPSCs bicuculline was not included; presynaptic action potentials were blocked with 200 nM TTX. All Purkinje cells studied were located at the surface of the slices. No cleaning of the cell surface was performed before recording. Recording pipettes (2–2·5 MΩ) were filled with a solution containing (mM): 150 CsCl, 4·6 $MgCl_2$, 0·1 $CaCl_2$, 1 EGTA, 10 Hepes acid, 0·4 Na-GTP and 4 Na-ATP (pH 7·3). Cancellation of capacitive current and series resistance compensation (nominally 60–70%) were performed. Uncompensated access resistance values during whole-cell recording were considered acceptable up to 12 MΩ. The holding potential was −60 mV. Spontaneous and miniature IPSCs were analysed off-line by using an event detection routine (Vincent & Marty, 1993). For

* To whom correspondence should be addressed.

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DSI experiments trains of depolarizing pulses were applied every 4 min. The results from the first stimulation were excluded from the analysis to minimize contamination with long-lasting postsynaptic potentiation (Llano *et al.* 1991). For each experiment the results were averaged over several trials to improve the accuracy of the evaluation of the percentage of inhibition. These mean values were then used to construct summary graphs showing percentages of reduction. Bars show means ± S.E.M. across experiments, and n is the number of experiments. A fraction of the cells failed to display DSI or inhibition of IPSCs by DCG-IV (16 and 17%, respectively). These cells were identified in the beginning of the recordings and the experiments were discontinued. In some experiments DCG-IV was applied with a pressure ejection pipette. The pipette had an opening of 2–3 μm and was positioned above the dendritic arborization of the Purkinje cell under study.

For $[Ca^{2+}]_i$ measurements, the internal solution was supplemented with 250 μM fura-2 (K^+ salt, Molecular Probes). The excitation wavelength was alternately switched between 356 and 390 nm. The fluorescence signal ($\lambda > 470$ nm) was collected from a 20 μm × 20 μm square area centred on the cell soma. $[Ca^{2+}]_i$ was calculated after subtraction of the background by using a calibration curve obtained from whole-cell recordings of Purkinje cells with solutions of known pCa values (see details in Fierro & Llano, 1996).

Bicuculline, 6-nitro-7-sulphamoylbenzo(*f*)quinoxaline-2,3-dione (NBQX), D-2-amino-5-phosphono-pentanoic acid (D-APV), D- and L-2-amino-3-phosphonopropionic acid (D- and L-AP3), (+)-α-methyl-4-carboxyphenylglycine ((+)-MCPG), and *trans*-1-amino-cyclopentane-1,3-dicarboxylate (*t*-ACPD) were purchased from Tocris Cookson (Bristol, UK). 2-(2,3-Dicarboxycyclopropyl)-glycine (DCG-IV) was a kind gift from Dr Y. Ohfune of the Suntory Institute of Bioorganic Research, Osaka, Japan. Other chemicals were purchased from Sigma.

RESULTS

The spontaneous synaptic activity recorded in Purkinje cells under standard conditions is mainly due to the input from interneurones of the molecular layer (Vincent *et al.* 1992). DCG-IV is a potent and highly specific agonist of group II metabotropic glutamate receptors (mGluRs), which comprises mGluR2 and mGluR3 (Ohfune, Shimamoto, Ishida & Shinozaki, 1993). Local applications of DCG-IV led to a strong reduction of the amplitude and frequency of sIPSCs (Fig. 1*A*). In the same cells, DSI can be measured as a reduction of the synaptic activity when applying a depolarizing train (Fig. 1*B*). The magnitude and recovery kinetics of the DCG-IV-induced effect, as well as the pattern of the inhibition in terms of amplitude and frequency, were strikingly similar to those obtained upon depolarization of the Purkinje cell (Fig. 1*C*). These results show that DCG-IV mimicks the effects of DSI on sIPSCs.

In the presence of TTX, bath application of DCG-IV, like that of the wide spectrum mGluR agonist *t*-ACPD (Llano & Marty, 1995), reduced the frequency of mIPSCs by almost half, with little effect on their mean amplitude. Again the pattern of inhibition was identical in the presence of DCG-IV and during DSI (Fig. 1*D*). These results indicate that GABA release in cerebellar interneurones is controlled by mGluR2,3 receptors, as previously shown in the olfactory bulb and hippocampus (Hayashi *et al.* 1993; Poncer, Shinozaki & Miles, 1995), and that activation of these receptors closely mimicks both TTX-blockable and TTX-independent components of DSI.

If DCG-IV-induced inhibition and DSI involve the same presynaptic receptors it should be possible to demonstrate competition between the two effects. Inhibitory effects of DCG-IV did not increase significantly with DCG-IV concentration between 1 and 5 μM (results not shown), indicating saturation of the corresponding mGluRs. DSI was significantly reduced in the presence of 0·5–5 μM DCG-IV, both in control saline and in the presence of TTX, indicating competition (Fig. 2*A* and *D*). Since DSI depends on a rise of the postsynaptic $[Ca^{2+}]_i$ (Llano *et al.* 1991; Vincent *et al.* 1992; Pitler & Alger, 1992), the possibility was considered that DCG-IV could inhibit the postsynaptic $[Ca^{2+}]_i$ rise during induction of DSI. Control experiments using the fluorescent dye Calcium Green showed that DCG-IV did not alter $[Ca^{2+}]_i$ signals elicited by depolarizing trains ($n = 5$; data not shown). Taken together, the results are consistent with the view that DSI and DCG-IV-induced inhibition compete for the same mGluRs.

mGluR2,3 receptors are negatively coupled to cAMP production (Pin & Bockaert, 1995). Furthermore, forskolin and β-adrenergic agonists increase the frequency of both sIPSCs and mIPSCs in Purkinje cells and thus have effects opposite to those of DSI (Llano & Gerschenfeld, 1993). We therefore hypothesized that DSI could involve an inhibition of adenylate cyclase and a subsequent drop in cAMP concentration in the presynaptic neurones. This hypothesis is consistent with the sensitivity of DSI to pertussis toxin in hippocampal slices (Pitler & Alger, 1994). We found that DSI was markedly smaller during treatment with forskolin than in control conditions (Fig. 2*B*). Two control experiments were performed. First it was shown that forskolin did not inhibit pulse-evoked $[Ca^{2+}]_i$ rises (Fig. 2*C*); actually it potentiated both the resting $[Ca^{2+}]_i$ and pulse-evoked $[Ca^{2+}]_i$ rises. On average the ratio of $[Ca^{2+}]_i$ values in forskolin over control was 2·02 ± 0·15 for the baseline and 1·31 ± 0·22 for the peak transient after the third pulse ($n = 4$). Secondly, enhancing transmitter release by increasing the bath K^+ concentration did not alter the extent of DSI, showing that the increase in synaptic activity brought about by forskolin was not responsible *per se* for the changes in DSI (Fig. 2*E*). These results suggest a link between cAMP and DSI, and are consistent with the view that activation of mGluR2,3 receptors during DSI induces an inhibition of adenylate cyclase.

Finally antagonists of mGluRs were used to test the involvement of mGluRs in DSI. The wide spectrum mGluR antagonist MCPG failed to block DSI. However L-AP3, an inhibitor of the effects of DCG-IV in the hippocampus (Poncer *et al.* 1995), reduced DSI (Fig. 3*A* and *C*). Again it was verified that L-AP3 did not alter postsynaptic $[Ca^{2+}]_i$

changes during DSI induction (Fig. 3*B*). On average the ratio of $[Ca^{2+}]_i$ values in L-AP3 over control was 1·27 ± 0·07 for the baseline and 0·96 ± 0·05 for the peak transient after the third pulse ($n = 5$). L-AP3 is a broad spectrum substance which acts on several subtypes of glutamate receptors. In the present preparation, application of L-AP3 led to an increase of the frequency of spontaneous or miniature IPSCs. This effect was inhibited by D-APV, suggesting that it was mediated by NMDA receptors. Application of L-AP3 also induced an inward current in Purkinje cells. NBQX, a selective blocker of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-selective glutamate receptors,

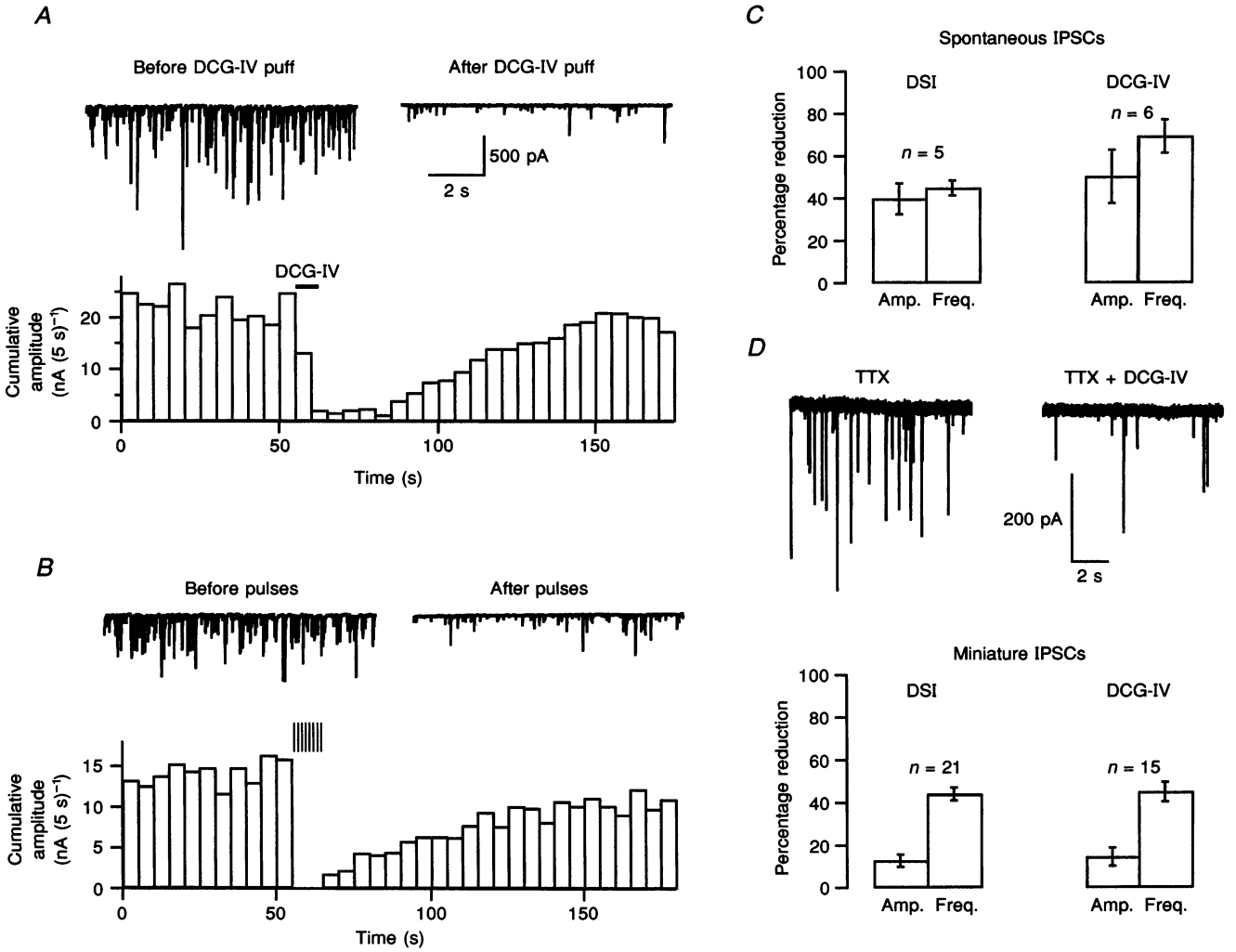

Figure 1. The mGluR agonist DCG-IV mimicks DSI

A, a puff application of DCG-IV (concentration in application pipette, 5 μM; 6 s pressure application indicated by bar above histogram) reduces the mean frequency and amplitude of sIPSCs. Upper trace, records obtained about 5 s before and after the DCG-IV puff. Lower plot, cumulated amplitudes of sIPSCs over 5 s time bins. The effects of DCG-IV are maximal a few seconds after the end of the pulse, and they slowly recover over a 2 min period. *B*, DSI reduces sIPSCs similarly to a DCG-IV puff. Same cell as in *A*. Upper traces, records obtained about 5 s before and after applying a series of depolarizing pulses (8 × 100 ms steps to 0 mV at 1 s intervals) to the recorded cell. Lower plot, cumulated amplitudes of sIPSCs over 5 s time bins. The position of the train is indicated above the histogram. Same calibrations in *A* and in *B*. *C*, summarized results from 6 experiments similar to that illustrated in *A* and *B*. In 5 of these experiments both DSI and DCG-IV application results were available. The control results were taken over a period of 60 s before applying the DCG-IV or the voltage pulses; the test results were taken over a period of 10 s starting 5 s after the end of the stimulus. On average DCG-IV reduced the main amplitude and frequency of sIPSCs similarly to DSI, even though the mean event frequency was slightly more reduced by DCG-IV than by DSI. *D*, DCG-IV and DSI have similar effects on mIPSCs. Upper traces: mIPSCs recorded before and during bath application of 1 μM DCG-IV, in the presence of TTX. Lower plots, left, analysis of the effects of DSI on the mean amplitude and frequency of mIPSCs; right, results of bath application of 1–5 μM DCG-IV.

significantly reduced the amplitude of the L-AP3-induced current from 344 ± 41 ($n = 18$) to 120 ± 18 pA ($n = 5$; $P < 0{\cdot}01$), suggesting the involvement of AMPA-selective receptors. Neither D-APV nor NBQX altered the level of DSI (results not shown). Moreover, the ability of L-AP3 to inhibit DSI was not affected by the presence of either or both blockers. Altogether the effects of L-AP3 on DSI were tested in twenty cells; ten of these experiments were performed in D-APV (10–50 μM) and six in the combined presence of D-APV and NBQX (10 μM). There was no significant difference between the results of the three groups, which were then pooled. On average DSI was reduced by L-AP3 from $51{\cdot}3 \pm 2{\cdot}6\%$ in the control to $30{\cdot}0 \pm 4{\cdot}4\%$, a highly significant reduction ($P < 0{\cdot}0002$).

These results indicate that L-AP3, independently of its inhibition of DSI, activates presynaptic NMDA-selective

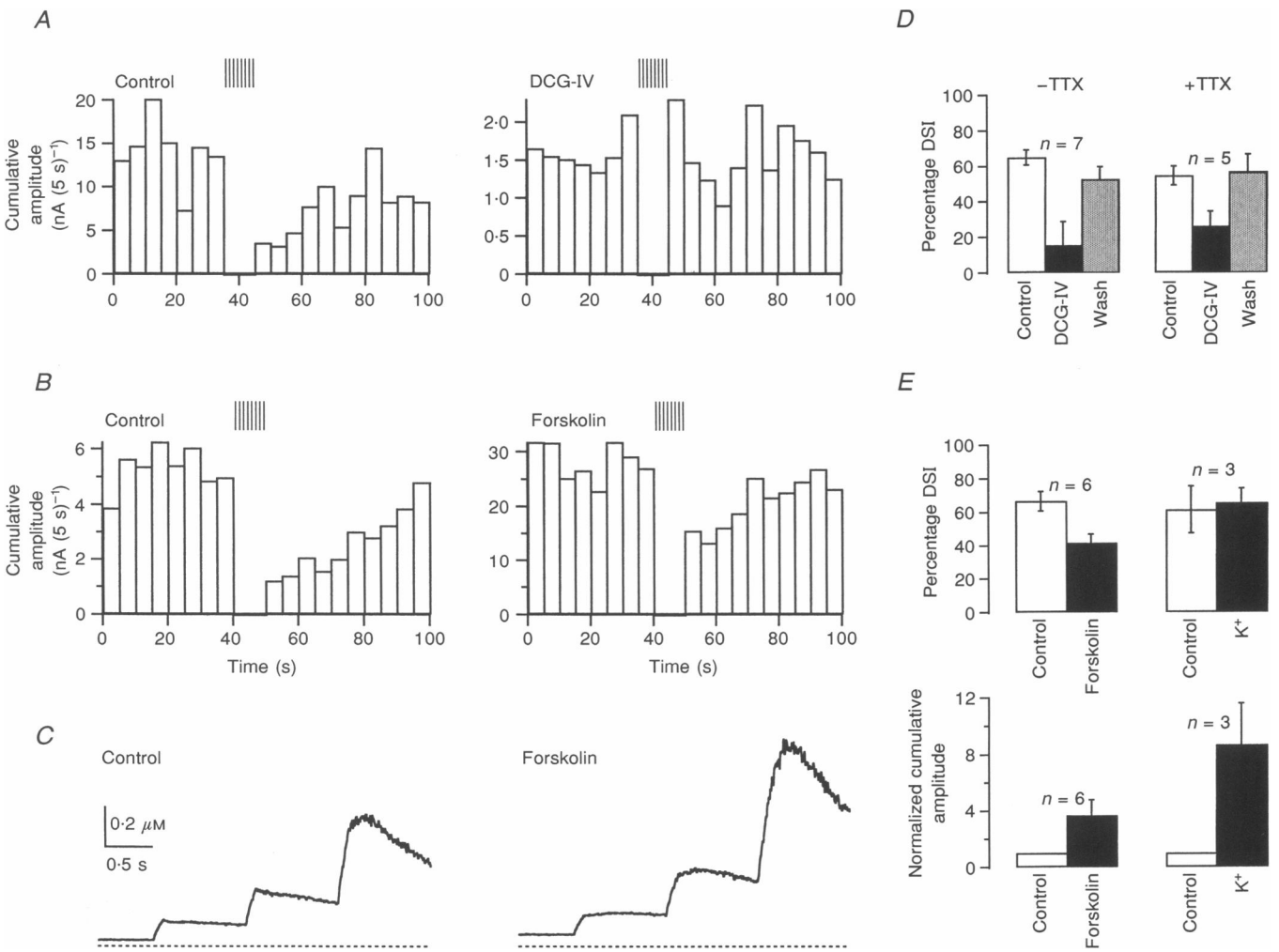

Figure 2. DCG-IV or forskolin application interfere with DSI

A, plots of summed sIPSCs over peristimulus periods in control saline (left) and after bath application of DCG-IV (0·5 μM, right; average of 2 trials). Each train of depolarizing pulses is indicated by a series of vertical bars. Note that the baseline level of summed IPSCs is reduced by DCG-IV about 10-fold. No DSI is detectable in the presence of DCG-IV. *B*, plots of summed sIPSCs over peristimulus periods in control saline (left; average of 2 trials) and in the presence of 20 μM forskolin (right; average of 2 trials). Application of forskolin potentiates the sIPSCs, and leads to a relative decrease of DSI. *C*, $[Ca^{2+}]_i$ measurements during the trains, from an experiment similar to that shown in *B*. Only the responses to the first 3 voltage pulses (out of 8) are shown because responses to later pulses exceeded the range of Ca^{2+} concentration where the fura-2 response is reliable. Dotted line, 0 Ca_i^{2+} level. *D*, summary of experiments as illustrated in *A*. Left, experiments performed in normal saline (sIPSCs; 0·5–1 μM DCG-IV). Right, experiments in TTX (mIPSCs; 3–5 μM DCG-IV). DCG-IV significantly reduced the percentage of DSI compared with the control value both in TTX-free saline ($P < 0{\cdot}02$) and in TTX ($P < 0{\cdot}05$). *E*, summary of experiments as in *C*, and of control experiments where the sIPSCs were enhanced by increasing extracellular K^+ concentration to 7·5–10 mM. Upper plots compare the percentage of DSI before and after application of the test solution (forskolin or high K^+). Forskolin significantly reduced the percentage of DSI ($P < 0{\cdot}01$). Lower plots show the extent of potentiation of sIPSCs due to forskolin and to high K^+.

and postsynaptic AMPA-selective glutamate receptors. In the presence of the two blockers of ionotropic glutamate receptors, L-AP3 also reduced the basal mIPSC frequency by 28 ± 17% ($P < 0.05$). These results suggest that L-AP3 acts both as an antagonist and as a partial agonist of the mGluRs involved in DSI.

Based on the above conclusion that DCG-IV and DSI activate the same receptors, the inhibitory effects of L-AP3 on DSI should be paralleled by a similar effect on DCG-IV-induced inhibition. The DCG-IV-induced inhibition of mIPSCs was therefore compared with and without L-AP3. Again the results were essentially the same whether experiments were performed in the presence of D-APV and/or NBQX or in the absence of these blockers, and all results were therefore pooled. On average the percentage of reduction of summed mIPSC amplitudes by DCG-IV (2 μM) decreased from 66·8 ± 4·7% in the control to 21·7 ± 6·7% in the presence of 1 mM L-AP3 ($P < 0.0001$). The results summarized in Fig. 3*C* show a parallelism between the pharmacological properties of DCG-IV-induced inhibition and DSI. In particular D-AP3, the stereoisomer of L-AP3, did not antagonize the effects either of DCG-IV or of DSI.

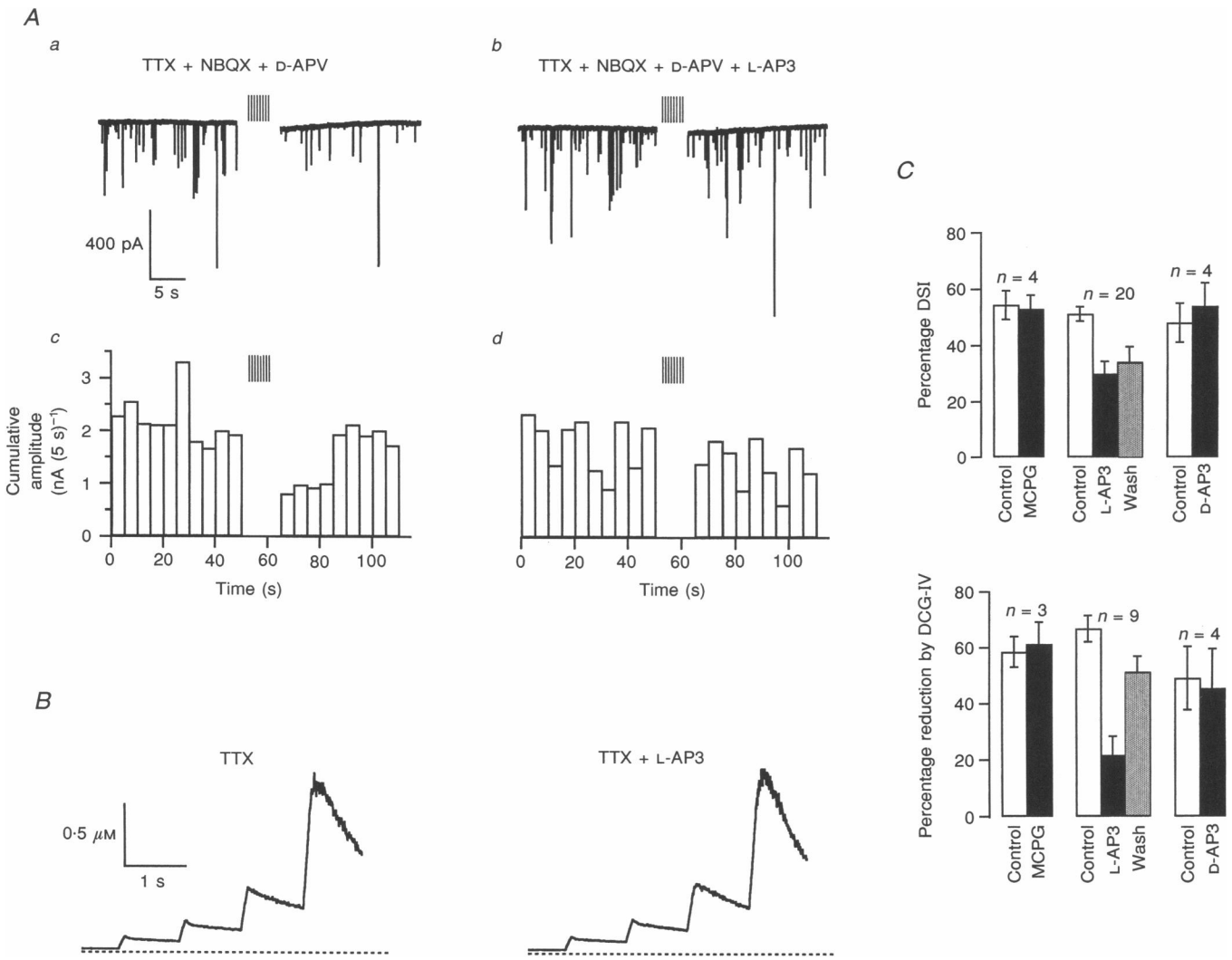

Figure 3. The mGluR antagonist L-AP3 inhibits DSI

A, results from 1 cell in 0·2 μM TTX, 10 μM NBQX and 50 μM D-APV. Upper traces, mIPSCs about 20 s before and 5 s after trains of depolarizing pulses (indicated by a series of vertical bars). *A a*, control; *A b*, in 1 mM L-AP3; *A c* and *A d*, cumulated amplitudes of mIPSCs over 5 s time bins. Averaged results from 3 trials (*c*) and from 2 trials (*d*). *B*, $[Ca^{2+}]_i$ signals from another cell in TTX, corresponding to the first 4 voltage pulses of each train. Left, control. Right, in 1 mM L-AP3. Dotted lines, 0 Ca_i^{2+} level. L-AP3 does not modify depolarization-induced $[Ca^{2+}]_i$ rises. *C*, summary of the sensitivity of DSI and of DCG-IV-induced inhibition to (+)-MCPG, L-AP3 and D-AP3. All experiments were performed in the presence of TTX. L-AP3 significantly reduces DSI, as well as DCG-IV-induced inhibition. On the other hand neither (+)-MCPG (1 mM) nor D-AP3 (1 mM) were able to modify the effects of DSI or of DCG-IV on summed mIPSC amplitudes.

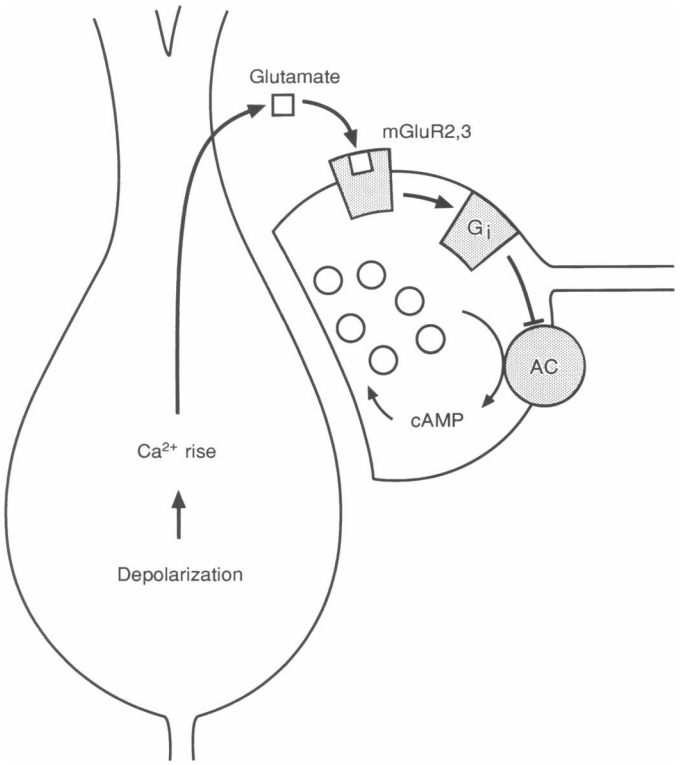

Figure 4. A working hypothesis for the mechanism of DSI

Depolarization-induced Ca^{2+} entry leads to the release of glutamate, or of a glutamate-like substance, to the extracellular space. This results sequentially in an activation of presynaptic mGluR2,3 receptors, an inhibition of adenylate cyclase (AC), a decrease of the presynaptic cAMP concentration and a decreased GABA release. mGluR2,3 and adenylate cyclase are supposed to be linked together by a pertussis toxin-sensitive GTP-binding protein (G_i), as suggested by data by Pitler & Alger (1994) and by the known properties of group II mGluRs (Pin & Bockaert, 1995). (For simplicity only the TTX-resistant component of DSI is depicted. In the absence of TTX, DSI also involves a modification of presynaptic electrical excitability (Marty & Llano, 1995).)

Likewise (+)-MCPG, which failed to inhibit DSI, also failed to inhibit the effects of DCG-IV, suggesting that it is not effective at the receptors involved here. Nevertheless the batches of (+)-MCPG used in the study were able to fully block at 1 mM the mGluR1-mediated inward current observed upon application of *t*-ACPD in Purkinje cells (Staub, Vranesic & Knöpfel, 1992; results not shown), suggesting a normal effectiveness on mGluRs.

DISCUSSION

The results strongly suggest that group II metabotropic glutamate receptors play a key role in DSI. The pattern of DCG-IV effects indicates, in agreement with *in situ* hybridization results (Ohishi, Shigemoto, Nakanishi & Mizuno, 1993), that these receptors are located on interneurones rather than on Purkinje cells. Therefore we propose that glutamate, or a glutamate-like substance, is the retrograde messenger of DSI. Because group II metabotropic glutamate receptors are negatively coupled to adenylate cyclase, we further propose that DSI involves a decrease in the presynaptic concentration of cAMP (Fig. 4). This proposal is consistent with the results of Fig. 2 showing interaction between DSI and forskolin-induced potentiation.

Even though Purkinje cells are GABAergic their cytosol, like that of all neurones, contains millimolar concentrations of glutamate (Attwell, Barbour & Szatkowski, 1993). How a glutamate-like substance could be released is, however, unclear. A mechanism based on reversed glutamate transport (Attwell *et al.* 1993) would be inconsistent both with the stoichiometry of known glutamate transporters and with the dependence of DSI on a postsynaptic $[Ca^{2+}]_i$ rise (Llano *et al.* 1991; Pitler & Alger, 1992; Vincent & Marty, 1993). It is more likely that release occurs by exocytosis. Glial cells and cholinergic neurones can secrete glutamate through Ca^{2+}-dependent vesicular release (Parpura, Basarsky, Liu, Jeftinija, Jeftinija & Haydon, 1994; Dan, Song & Poo, 1994), presumably by activation of the constitutive exocytosis pathway.

Because of the many similarities between DSI in Purkinje and in CA1 pyramidal cells (Alger & Pitler, 1995; Marty & Llano, 1995), the present results raise the possibility that glutamate or a glutamate-like substance is released in the somato-dendritic region of CA1 pyramidal cells following voltage-dependent Ca^{2+} entry. Since the induction of long-term potentiation (LTP) in the same cells involves a postsynaptic $[Ca^{2+}]_i$ rise as an obligatory step, it is tempting to speculate that the same substance could be the long-sought retrograde messenger implicated in LTP (Bliss & Collingridge, 1993).

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Author's email address

A. Marty: amarty@gwdg.de

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