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**Protease treatment of cerebellar Purkinje cells renders ω -Agatoxin IVA-sensitive Ca^{2+}
channels insensitive to inhibition by ω -conotoxin GVIA.**

Elizabeth W. Tringham, Jonathan R.B. Dupere, C. Elizabeth Payne & Maria M. Usowicz

Dept. of Pharmacology, University of Bristol, University Walk, Bristol, BS8 1TD UK (E.W.T.,
J.R.B.D., C.E.P., M.M.U.)

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Corresponding author:

Dr Maria M. Usowicz,

Dept. of Pharmacology, University of Bristol, University Walk, Bristol BS8 1TD, UK.

Tel: 44(0)117-331-1457 Fax: 44(0)117-331-2288 E-mail: m.m.usowicz@bris.ac.uk

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Abstract

The identification of currents carried by N-type and P-type Ca^{2+} channels in the nervous system relies on the use of ω -conotoxin GVIA (ω -CTx-GVIA) and ω -Agatoxin IVA (ω -Aga-IVA). The peptide ω -Aga-IVA inhibits P-type currents at nanomolar concentrations and N-type currents at micromolar concentrations. ω -CTx-GVIA blocks N-type currents but there have been no reports that it can also inhibit P-type currents. To assess the effects of ω -CTx-GVIA on P-type channels, we made patch-clamp recordings from the soma of Purkinje cells in cerebellar slices of mature (postnatal days 40-50, P40-50) and immature (P13-20) rats, in which P-type channels carry most of the Ca^{2+} channel current ($\geq 85\%$). These showed that micromolar concentrations of ω -CTx-GVIA inhibited the current in P40-50 cells (66%, 3 μM ; 78%, 10 μM) and in P13-20 Purkinje cells (86%, 3 μM ; 89%, 10 μM). The inhibition appeared to be reversible, in contrast to the known irreversible inhibition of N-type current. Exposure of slices from young animals to the enzyme commonly used to dissociate Purkinje cells, protease XXIII, abolished the inhibition by ω -CTx-GVIA but not by ω -Aga-IVA (84%, 30 nM). Our finding that micromolar concentrations of ω -CTx-GVIA inhibit P-type currents suggests that specific block of N-type current requires the use of submicromolar concentrations. The protease-induced removal of block by ω -CTx-GVIA but not by ω -Aga-IVA indicates a selective proteolytic action at site(s) on P-type channels with which ω -CTx-GVIA interacts. It also suggests that Ca^{2+} channel pharmacology in neurons dissociated using protease may not predict that in neurons not exposed to the enzyme.

Introduction

Voltage-gated Ca^{2+} channels control numerous cellular functions, including neuronal electrical activity, gene expression and intracellular signalling. The use of toxins originating from venoms of assorted species has aided the classification of Ca^{2+} channels as T-, N-, L-, P-, Q- or R-type, and helped elucidate the diverse physiological roles of the different Ca^{2+} channel classes (McDonough, 2004; McGivern, 2006). It has also helped implicate altered function of distinct types of Ca^{2+} channels in specific pathophysiological conditions and led to the development of an ω -conotoxin as an N-type channel blocker that is used in the management of intractable pain (Snutch, 2005; McGivern, 2006).

One characteristic by which N-type channels in neurons have been identified, and by which recombinant channels containing a $\text{Ca}_v2.2$ subunit have been matched to native N-type channels, is the irreversible inhibition of Ca^{2+} or Ba^{2+} currents by ω -conotoxin GVIA (ω -CTx-GVIA) (McDonough, 2004; McGivern, 2006). The property used to identify native P-type channels and recombinant channels containing a $\text{Ca}_v2.1$ subunit has been the irreversible inhibition of currents by ω -Agatoxin IVA (ω -Aga-IVA) (McDonough, 2004; McGivern, 2006). The selectivity of ω -Aga-IVA for P-type over N-type channels is not absolute; at concentrations higher than those necessary to block P-type currents, ω -Aga-IVA also blocks N-type currents (Sidach and Mintz, 2000). Conversely, the inhibition of P-type currents by ω -CTx-GVIA at concentrations above those that inhibit N-type currents has not been described. Prototypical P-type currents in the soma of enzymatically-dissociated cerebellar Purkinje neurons have been reported to be insensitive to ω -CTx-GVIA (Regan, 1991; Mintz et al., 1992). At odds with these electrophysiological studies are binding studies that provide evidence for the binding of ω -CTx-GVIA to P-type channels in

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rat brain membranes, albeit at concentrations many times higher than those that bind N-type channels (Lewis et al., 2000). Since an ω -conotoxin selective for N-type channels is used in the treatment of severe chronic pain (ω -conotoxin MVIIA, ω -CTx-MVIIA, also known as ziconotide) (Snutch, 2005), clarification of the effects of ω -conotoxins at P-type channels is of clinical significance. Inhibition of P-type currents may help explain some of the side-effects associated with the use of ω -CTx-MVIIA that cannot be explained by the inhibition of N-type channels (Snutch, 2005; McGivern, 2006). For these reasons we reexamined the sensitivity of P-type currents in cerebellar Purkinje cells to ω -CTx-GVIA by patch-clamp recording from cells in slices of mature (P40-50) and immature (P13-20) cerebellum.

The cerebellar Purkinje neuron is the cell-type of choice for an investigation of the effects of ω -CTx-GVIA on P-type currents because P-type channels carry most of the Ca^{2+} channel current whereas N-type channels are absent or mediate a minor current component. The current in the soma is inhibited in both immature and adult cells by concentrations of ω -Aga-IVA that are selective for P-type channels (80-85%, $IC_{50} < 1-3$ nM) over Q-type and N-type channels (Mintz et al., 1992; Sather et al., 1993; Sidach and Mintz, 2000; Tringham et al., 2007) and by ω -CTx-MVIIC with characteristically slow kinetics (McDonough et al., 1996; Tringham et al., 2007). A paucity of N-type current in the soma of cerebellar Purkinje cells is indicated by the slow kinetics of block by ω -CTx-MVIIC, by the lack of inhibition in adult Purkinje cells by submicromolar concentrations of ω -CTx-GVIA (Tringham et al., 2007) that are known to inhibit N-type currents (Boland et al., 1994; Bleakman et al., 1995; Lin et al., 1997), by inhibition of only a minor current component (0-5%) in dissociated immature cells by ω -CTx-GVIA (Regan, 1991; Mintz et al., 1992), and by the absence or weak expression of $\text{Ca}_v2.2$ mRNA (Tanaka et al., 1995;

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Volsen et al., 1995) or Cav2.2 protein (Volsen et al., 1995; Chung et al., 2000), although one study did detect Cav2.2 protein in Purkinje cells (Westenbroek et al., 1992).

We find that micromolar concentrations of ω -CTx-GVIA inhibit ω -Aga-IVA-sensitive channels in the soma of both immature and mature Purkinje cells in cerebellar slices, but not in immature cells exposed to protease XXIII. These findings suggest that in experiments investigating the pharmacological identity of Ca²⁺ channels, some P-type currents may be misclassified as N-type, unless submicromolar concentrations of ω -CTx-GVIA are used to block N-type channels. Inhibition of P-type channels by ω -CTx-GVIA may help explain the overlapping sensitivity of neurotransmission at brain synapses to ω -CTx-GVIA and ω -Aga-IVA, which is usually explained by a non-linear relationship between presynaptic Ca²⁺ concentration and transmitter release. Our findings also suggest that the sensitivity of Ca²⁺ channels to different compounds can be differentially altered by proteolysis.

Methods

Cerebellar slices

Parasagittal slices of cerebellar vermis (250-300 μm) were prepared from male Wistar rats as previously described (Usowicz et al., 1992; Tringham et al., 2007). Adult (P40-50) or young rats (P13-20) were culled by cervical dislocation and decapitated, in accordance with the United Kingdom Animal (Scientific Procedures) Act (1986) and with the University of Bristol Ethical Review Committee. The cerebellar vermis was excised and cut into slices in ice-cold oxygenated Krebs-Henseleit solution (composition in mM: 124 NaCl, 1.3 MgSO_4 , 5 KCl, 2.4 CaCl_2 , 1.2 KH_2PO_4 , 26 NaHCO_3 , 10 D-glucose, pH 7.4, bubbled with 95% O_2 / 5% CO_2) using a Vibratome (Series 1000, Pelco, Redding, CA) or a Leica VT100S vibrating microtome (Leica Microsystems, Nussloch, Germany). Slices were kept at room temperature for 1 - 7 hours before recording.

For some experiments, slices were exposed to the enzyme protease type XXIII (Sigma-Aldrich Co. Ltd., Poole, UK). Between 30 and 50 minutes after 6-8 slices had been cut, half of the slices were introduced into a beaker filled with Krebs solution containing 3 mg ml^{-1} protease XXIII. The remaining slices were placed in another beaker containing standard Krebs solution. Both beakers were bubbled with 95% O_2 / 5% CO_2 and maintained at 37°C for 7 minutes by immersion in a water bath. The solution in the beakers was then aspirated off and replaced with a Krebs solution containing 1 mg ml^{-1} trypsin inhibitor (type I-S, Sigma) and 1 mg ml^{-1} bovine serum albumin (Sigma). This was constantly bubbled with 95% O_2 / 5% CO_2 . The slices were then transferred to individual vials containing trypsin inhibitor and bovine serum albumin. After 1 hour, the solution was aspirated off and replaced with standard, oxygenated Krebs solution, in which the slices were then stored at room temperature for 1.5 – 3 hours before recording.

Cell-attached recording

Individual slices were viewed on a FS Axioskop microscope (Carl Zeiss, Welwyn Garden City, UK) and superfused ($1\text{--}1.5\text{ ml min}^{-1}$) with oxygenated (100% O_2) HEPES-buffered saline solution (composition in mM: 133 NaCl, 2.5 KCl, 2.4 CaCl_2 , 10 D-glucose, 20 HEPES, 1.3 MgCl_2 , pH 7.4 with NaOH, plus 1 μM tetrodotoxin (TTX)) at 21–25°C. Purkinje cells were identified by their relatively large soma (diameter $\sim 20\text{ }\mu\text{m}$), shape (single dendrite emerging from the soma) and location (at the interface of the molecular and granule cells layers; photograph in Tringham et al., 2007). Prior to recording with an Axopatch 200A amplifier (Axon Instruments, Union City, CA), the soma of a Purkinje cell was ‘cleaned’ by applying extracellular solution from a pipette (tip diameter, $\sim 5\text{ }\mu\text{m}$). Patch pipettes (thick-walled borosilicate glass capillaries, Harvard Apparatus, Kent, UK) were backfilled with a filtered ($0.2\text{ }\mu\text{m}$, cellulose acetate membrane) solution containing, in mM: 5 BaCl_2 , 10 CsCl, 10 HEPES, 134 or 150 TEA-Cl, 0.1 EGTA, pH 7.4 with TEA-OH, plus 1 μM TTX. Ca^{2+} channel currents were recorded from the soma of Purkinje cells in the cell-attached configuration, as previously described in detail (Tringham et al., 2007). Channels were opened by applying a depolarising voltage ramp to the pipette ($0 \rightarrow -170$ or -160 mV , 0.53 mV ms^{-1}) and closed with a repolarising voltage ramp (-170 or $-160 \rightarrow 0\text{ mV}$, 0.53 mV ms^{-1} , holding potential 30–50 mV negative to the cell resting potential). The ramps were applied at 0.2 Hz for 2.5 – 7.5 minutes, using a Cambridge Electronic Design (CED) 1401 *plus* A/D interface and CED patch and voltage-clamp software (v. 6.37, Cambridge, UK). Currents were low-pass filtered at 10 kHz (4 pole Bessel filter on the amplifier) and then at 2.04 kHz (8 pole low-pass Bessel filter, Frequency Devices, Haverhill, MA, USA), and acquired at 7 kHz. At the end of cell-attached recording, a whole-cell

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configuration was established in order to measure the resting membrane potential (TTX in the extracellular solution prevented spontaneous spiking). Patch-potentials (e.g. ~ -100 to $+70$ mV) were calculated as the resting cell potential (e.g. -60 mV) minus the pipette holding potential (e.g. $+40$ mV) and minus the applied voltage ramp ($0 \rightarrow -170$ mV or -160 mV). They were not corrected for the liquid junction potential between the bath solution and the pipette solution, which was only ~ -1 mV. Any inaccuracy in the measurement of the cell resting potential does not affect the findings presented here, because the analysis of the cell-attached currents consisted of measurement of the size of the peak current, irrespective of the potential at which the peak occurred.

Further analysis and subtraction of a linear leak current were performed off-line with Origin v. 6 (Microcal, Northampton, MA, USA). As previously described (Tringham et al., 2007), currents evoked by 15-30 ramps were averaged to give the mean current for each patch. A leak current was then estimated by fitting a straight line to the linear part of the mean current. It was subtracted from the mean current, to give the Ca^{2+} channel current carried by 5 mM Ba^{2+} for each patch, which was measured at the peak (I_{pk}).

Concentration-inhibition curves

The pharmacology of Ca^{2+} channels in the soma of mature Purkinje cells was investigated by recording currents in cell-attached patches with drug-free and drug-containing pipette solutions (Usowicz et al., 1992; Tringham et al., 2007). The solutions were introduced in to the pipettes using glass Pasteur pipettes pulled to a fine tip. Spontaneous firing of the Purkinje cells was prevented by the inclusion of $1 \text{ }\mu\text{M}$ TTX in the bathing and pipette solutions, and potassium

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currents were inhibited by Ba^{2+} , TEA^+ and Cs^+ in the pipette solution. Recordings in the presence or absence of a drug were interleaved throughout the day so as to ensure that any drug-induced differences in I_{pk} were not due to differences in current size between different slices or animals. For each cell-attached recording with a drug-free pipette solution, currents were evoked by 30 depolarising voltage ramps. Similarly, for each recording with a drug-containing pipette solution, 30 ramps were applied to each patch, so as to check that a drug-induced change in peak current had reached steady state within the 2.5 minutes of recording. Only currents which did not differ in size, usually over 15-30 ramps, were averaged in order to get a measure of the peak Ca^{2+} channel current, I_{pk} for that patch (Tringham et al., 2007). The sizes of I_{pk} in different patches in the absence of drug and in the presence of the same drug concentration are summarised as mean $\pm$ S.E.M.. The relationship between I_{pk} and log drug concentration was fitted by a logistic curve, of the form, $((\text{max}-\text{min})/(1 + ([\text{drug}]/IC_{50})^{nH}) + \text{min}$, where max is the maximum I_{pk} in the absence of drug, min. is the minimum current remaining, i.e. the current not blocked by the drug, IC_{50} is the molar drug concentration that produces 50% block of the sensitive current and nH is the Hill slope. The fit was weighted by the inverse of the square of the S.E.M at each point. Statistical significance was calculated using a two-tailed unpaired Student's t test, or one-way ANOVA followed by Dunnett's test, with significance taken at the $P < 0.05$ level.

Reversibility of the effects of ω -CTx-GVIA

ω -CTx-GVIA was applied to the cleaned soma of a Purkinje cell by pressure ejection for 5 minutes from a micropipette that was positioned as close as possible to the soma and whose diameter was greater than that of the soma (20 μM in micropipette). The force with which the solution flowed over the cell was sufficiently strong to cause distortion of the shape of the cell

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and movement of nearby cell debris. After 5 minutes, the micropipette was removed and the slice was perfused with standard extracellular HEPES-based solution for 4-8 minutes, to allow for unbinding of ω -CTx-GVIA, should it occur on this timescale. A cell-attached recording of Ca^{2+} channel currents was then made from the soma. In control experiments made from the same slices, a micropipette was filled with drug-free bath solution.

Whole-cell recording

Whole-cell voltage-clamp recordings were made from Purkinje cells as described previously (Tringham et al., 2007). Briefly, slices were superfused with HEPES-buffered saline. For recordings from P13-20 animals, this contained 10 μM SR 95531 hydrobromide and 20 μM Na_2CNQX in order to inhibit spontaneous synaptic currents, which were more frequent than in mature cells. Patch-pipettes (thin-walled borosilicate glass capillaries with an inner filament, Harvard Apparatus, Kent, UK) were backfilled with a filtered solution containing, in mM: 100 CsCl, 10 HEPES, 4.5 MgCl_2 , 4 MgATP , 14 phosphocreatine-di Tris, 0.3 GTP-Tris, 10 EGTA, pH 7.2 with TEA-OH. The pipette shanks were coated with Sylgard resin and pipette tips were polished down to resistances of $\sim 1.25 \text{ M}\Omega$. The first component of the two-component whole-cell capacitance transients evoked by 5 mV voltage jumps, which likely represent the somatic capacitance and the dendritic capacitance, was cancelled ($27.6 \pm 0.9 \text{ pF}$, mature Purkinje cells, $n = 17$; $26.8 \pm 1.2 \text{ pF}$, immature Purkinje cells, $n = 13$). The series resistance compensation and correction were set at 85-95% with a 10 μs lag. A depolarising voltage ramp ($-100 \text{ mV} \rightarrow \sim +70 \text{ mV}$, 0.55 mV ms^{-1}) followed immediately by a repolarising voltage ramp ($+70 \text{ mV} \rightarrow -100 \text{ mV}$, 0.55 mV ms^{-1}) was applied at 0.05 Hz. The cell was superfused with the HEPES-based solution supplemented with 1 μM TTX, in which 2.4 mM CaCl_2 was replaced by 2 mM BaCl_2 , from a

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needle (made of polyimide, 360 μm in diameter, Digitimer Ltd., Welwyn Garden City, UK) placed under the water immersion objective ~ 0.5 cm away from the soma. The main extracellular cation was Na^+ and not TEA^+ as in the cell-attached recordings. Under these conditions, Ca^{2+} channel currents were recorded in the presence of an outward current (see Tringham et al., 2007). Currents were low-pass filtered at 2 kHz (10 kHz, 4 pole Bessel filter on the amplifier, followed by 2.04 kHz, 8 pole low-pass Bessel filter, Frequency Devices, Haverhill, MA, USA), acquired at 6.2 kHz and analysed off-line by CED patch and voltage-clamp programs. Further analysis was performed with Origin v. 6 (Microcal, Northampton, MA, USA).

The effect of ω -CTx-GVIA on whole-cell Ca^{2+} channel currents in mature cells was examined by switching the solution flowing out of the 360 μm needle from a drug-free to a drug-containing '2 mM Ba^{2+} extracellular solution', which also contained 1 μM TTX. The needle was connected via a multibarrel manifold (Digitimer Ltd.) and Teflon tubing and valves to solutions stored in glass syringes. The effect of ω -CTx-GVIA on whole-cell Ca^{2+} channel currents in immature cells was examined by lowering a drug-containing micropipette (~ 10 μm in diameter) into the bath and positioning it $\sim 20 - 60$ μm from the soma. The bath perfusion was switched off, the flow of the 2 mM Ba^{2+} solution out of the needle was stopped, and drug-containing, 2 mM Ba^{2+} -TTX solution, was forced out of the micropipette on to the soma by applying mouth pressure to the micropipette via a piece of tubing. The pressure applied was adjusted so that there was no visible damage or distortion of the cell since this can increase the series resistance and consequently reduce the size of the current recorded (Tringham et al., 2007). Recordings were terminated if the series resistance increased above 10 $\text{M}\Omega$ (mean value before 85-95%

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compensation: $3.7 \pm 0.3 \text{ M}\Omega$, mature Purkinje cells, $n = 17$; $5.3 \pm 0.5 \text{ M}\Omega$, immature Purkinje cells, $n = 13$).

Drugs

ω -CTx-GVIA and ω -Aga-IVA (Scientific Marketing Associates, Beckenham, UK, or Peptide Institute, Japan) were prepared as stock solutions of, respectively, 1-3 mM in sterile degassed water (degassed by vacuum for approximately 30 minutes and then perfused with nitrogen for a further 30 minutes) or 100 μM in filtered Milli-Q water. They were stored as aliquots at -80°C . For experiments in which drugs were included in the cell-attached recording electrode, further dilutions were made with the '5 mM Ba^{2+} pipette' solution and stored as single-use aliquots at -80°C . For experiments in which ω -CTx-GVIA was applied from a micropipette during whole-cell recording, a concentration of 20 μM was made from a stock aliquot, in the HEPES-based extracellular solution, on the day of recording.

Results

Inhibition of P-type Ca^{2+} channel currents in cell-attached patches of the soma of mature Purkinje cells by ω -CTx-GVIA

Pharmacological characterization of Ca^{2+} channel currents in the soma of adult rat cerebellar Purkinje cells (P40-50) has determined that most of the current ($\geq 85\%$) is carried by P-type channels and there is little or no contribution from L-type or N-type channels (Tringham et al., 2007). The susceptibility of these channels to block by ω -CTx-GVIA, a widely-used irreversible blocker of N-type channels, was investigated by recording macroscopic Ca^{2+} channel currents carried by 5 mM Ba^{2+} in somatic cell-attached patches of Purkinje cells in cerebellar slices, with pipettes containing different concentrations of ω -CTx-GVIA. The toxin was included in the pipette so as to avoid uncertainty about the toxin concentration at the channels of interest that can arise during bath administration as a result of non-specific binding of peptide toxins to the perfusion system and the slice (McDonough, 2004; Tringham et al., 2007). As described previously (Tringham et al., 2007), currents were evoked with depolarising voltage ramps (Fig. 1A, upper trace) and corrected for a linear leak current to reveal the Ca^{2+} channel current, which was measured at its peak, I_{pk} . Figure 1 A-C shows superimposed traces of currents recorded in many patches with drug-free pipettes (Fig. 1A) and with pipettes containing 3 μM (Fig. 1B) or 20 / 30 μM (Fig. 1C) ω -CTx-GVIA. They portray inhibition of the channels by micromolar concentrations of ω -CTx-GVIA. The plot of the mean I_{pk} for currents evoked in the presence of different concentrations of ω -CTx-GVIA (Fig. 1D) displays a maximal decrease of 80%. The logistic fit yields an IC_{50} of 1.6 μM , but this is only an approximate value, given the large errors associated with the mean values at 100 nM and 1 μM .

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The inhibition of native N-type channels (McCleskey et al., 1987; Aosaki and Kasai, 1989; Plummer et al., 1989; Boland et al., 1994) and recombinant Ca_v2.2-containing channels (Williams et al., 1992; Fujita et al., 1993; Stea et al., 1993; Bleakman et al., 1995) by ω -CTx-GVIA is irreversible. This was detected in early studies of the actions of ω -CTx-GVIA as persistent inhibition of N-type Ca²⁺ channel currents carried by 2.5 or 20 mM Ba²⁺ or 10 mM Ca²⁺, after cessation of application of ω -CTx-GVIA from a micropipette containing 5–16 μ M (McCleskey et al., 1987; Aosaki and Kasai, 1989; Plummer et al., 1989). By contrast, we found that local application of ω -CTx-GVIA to the cell soma for 5 minutes by pressure ejection from a micropipette containing 20 μ M, followed by 4–8 minutes wash, did not affect the size of the currents subsequently-recorded (Fig. 1E). The lack of irreversible inhibition confirms that most of the current in the soma of mature Purkinje cells is not carried by N-type channels. It also suggests that the block of P-type channels is reversible, but since the concentration required in the recording pipette for maximal block during cell-attached recording is $\sim$ 10 μ M (Fig. 1D) and the micropipette contained 20 μ M, we cannot exclude the possibility that the concentration reaching the soma during local perfusion did not cause maximal block and it was a partial block that reversed upon washing.

It is known that at least 85% of the Ca²⁺ channel current in the soma of adult Purkinje neurons is inhibited by ω -Aga-IVA (Tringham et al., 2007) while the experiments described so far suggest that $\sim$ 80% is inhibited by ω -CTx-GVIA. It is possible, therefore, that at least 65% of the current is carried by channels that can be inhibited by both compounds and the remainder is carried by two populations of channels, one sensitive to ω -Aga-IVA and the other sensitive to ω -CTx-GVIA. Alternatively, a major proportion of the current is inhibited by both compounds and the

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remaining minor component is insensitive to both. We tested these possibilities by including both ω -Aga-IVA and ω -CTx-GVIA in the recording pipettes. Initially, 30 nM Aga-IVA, which produces maximal inhibition (Tringham et al., 2007), was combined with a concentration of ω -CTx-GVIA, 100 nM, that by itself had no effect on the channels (Fig. 1D). This combination caused only 40% inhibition (Fig. 1F) which is less than the ~91% inhibition caused by ω -Aga-IVA alone (Fig. 1F). The occlusion of the inhibition by ω -Aga-IVA demonstrates that ω -CTx-GVIA binds to the same channel as ω -Aga-IVA. Elevating the concentration of ω -CTx-GVIA in the mixture to 30 μ M increased the inhibition to ~94% (Fig. 1F). This was similar to the ~91% inhibition caused by ω -Aga-IVA alone (Fig. 1F) but was more than the ~80% inhibition caused by ω -CTx-GVIA alone (Fig. 1D). These data suggest that a small fraction of the current is insensitive to both drugs (6-9%), a small fraction (~14%) can be inhibited by ω -Aga-IVA but not by ω -CTx-GVIA, and the majority of the current (~80%) can be blocked by ω -CTx-GVIA or ω -Aga-IVA.

Inhibition of whole-cell Ca^{2+} channel currents in the soma of adult Purkinje cells during superfusion of ω -CTx-GVIA.

It is difficult to predict from our cell-attached recordings with drug-free and drug-containing pipettes, the effect that ω -CTx-GVIA would have on brain slices in experiments requiring acute toxin application, since the concentration reaching the cell or synapse of interest may be much reduced after adsorption to the slice, the recording chamber or the tubing used to deliver the drug, (McDonough, 2004). To determine if inhibition of Ca^{2+} channels in Purkinje cells can be detected during application of ω -CTx-GVIA to a cerebellar slice, we recorded whole-cell Ba^{2+} (2 mM) currents evoked by voltage ramps (Tringham et al., 2007) and applied 3 μ M ω -CTx-GVIA.

Binding of the toxin to the perfusion system and to the slice was minimized by using tubes made of Teflon that were attached to a needle made of polyimide (360 μm diameter), and by placing the needle under the immersion objective, near the soma (~ 0.5 cm). Figure 2 shows that the whole-cell peak current, I_{pk} , was reduced by $\sim 25\%$ during 20 minutes of application (corrected for rundown by comparison with currents recorded in drug-free solution). For reasons of cost, the application was not continued until maximal block was reached. It is notable that the rate of block was slow and that the level of inhibition after 20 minutes was far less than that caused by the same concentration in cell-attached recordings (Fig. 1).

Inhibition of P-type Ca^{2+} channel currents in cell-attached patches of the soma of young Purkinje cells by ω -CTx-GVIA

Our finding that ω -CTx-GVIA inhibited $\sim 85\%$ of the ω -Aga-IVA-sensitive Ca^{2+} channel current in mature Purkinje cells (80% of total current) is in marked contrast to previous reports that 3-5 μM ω -CTx-GVIA inhibits ω -Aga-IVA-sensitive Ca^{2+} channel currents in cerebellar Purkinje cells by, at most, 5% (Regan, 1991; Mintz et al., 1992). To determine if this discrepancy might reflect the younger ages of the Purkinje cells examined in the previous studies (P7-21), we investigated the effects of ω -CTx-GVIA on Ca^{2+} channels in the soma of Purkinje cells in cerebellar slices obtained from P13-20 rats. Concentrations of ω -CTx-GVIA that caused maximal or near-maximal inhibition in mature cells also decreased the size of the currents recorded in cell-attached patches of immature cells, by 86% at 3 μM and 97% at 20 μM (Fig. 3A and 3B). We confirmed that most of the current (78%, Fig. 3C) was also inhibited by 30 nM ω -Aga-IVA (Mintz et al., 1992; Tringham et al., 2007). Local application of ω -CTx-GVIA to the cell soma for 5 minutes from a micropipette containing 20 μM , followed by 3-8 minutes

wash, did not affect the size of the currents subsequently-recorded (Fig. 3D). The lack of an irreversible decrease confirms that, as in mature cells, most of the current in immature Purkinje cells is not carried by N-type channels (Regan, 1991; Mintz et al., 1992). It also suggests that the inhibition of P-type channels is reversible, although as discussed above, it may be that the application from the micropipette caused only a partial block that reversed upon washing.

Inhibition of whole-cell Ca^{2+} channel currents in the soma of young Purkinje cells during local application of ω -CTx-GVIA from a micropipette.

To confirm the inhibition of Ca^{2+} channels in immature Purkinje cells by ω -CTx-GVIA, we recorded whole-cell Ca^{2+} channel currents (2 mM Ba^{2+} as charge carrier) and applied ω -CTx-GVIA from a micropipette placed near the soma. This method of application should avoid 'loss' of toxin to adsorptive sites and minimize the amount of toxin required. To avoid false effects that can arise from distortion of the cell by the force with which the solution is applied, the toxin was applied from a micropipette with a tip diameter no bigger than 10 μm , under weak mouth pressure (see methods). Figure 4A shows that for a micropipette containing 20 μM ω -CTx-GVIA, this protocol decreased the currents by ~25% during 20-25 minutes of application. This is a markedly smaller inhibition than the >90% inhibition effected by inclusion of 20 μM ω -CTx-GVIA in pipettes used for cell-attached recording (Fig. 3A and B). The difference is consistent with our previous demonstration using ω -Aga-IVA as the channel blocker, that this method of application delivers a concentration of drug to the soma that is many times lower than that in the micropipette (Tringham et al., 2007). To investigate the reversibility of the block by ω -CTx-GVIA, we tried removing the micropipette and perfusing the slice with toxin-free solution. In only two recordings were we able to do this without affecting the holding current or series

resistance. As exemplified by the time-course from one cell in Fig. 4B, these suggested that the inhibition was reversible, once rundown was taken into account, but further experiments are required to confirm this finding.

Somatic Ca^{2+} channels in Purkinje cells exposed to protease are not inhibited by ω -CTx-GVIA

Since a difference in the age of the cells does not explain why we, but not others, find inhibition of somatic Ca^{2+} channels of cerebellar Purkinje neurons by ω -CTx-GVIA, we considered if this discrepancy might reflect the fact that the previous recordings were from young cells (P7-21) treated with enzyme during dissociation (Regan, 1991; Mintz et al., 1992; McDonough et al., 1996; Sidach and Mintz, 2000), whereas our recordings were from young cells (P13-20) that had not been exposed to enzymes. Prior to cell-attached recording, we treated cerebellar slices from young animals with the enzyme most commonly used to dissociate Purkinje cells, protease type XXIII (see methods) (Mintz et al., 1992; McDonough et al., 1996; Sidach and Mintz, 2000). Cell-attached recording from the protease-treated cells was more difficult than from cells in 'control slices' that had not been exposed to protease. Only currents recorded from cells with resting potentials more negative than -50 mV were included in the analysis (measured after cell-attached recording). Currents recorded with drug-free pipettes from protease-treated cells are shown in Fig. 5A. The mean I_{pk} of these currents was not significantly different from the mean I_{pk} measured in control slices (Fig. 5B). Notably, the currents recorded with $3 \mu\text{M}$ ω -CTx-GVIA in the pipette from protease-treated slices were not different in size from control currents (Fig. 5B). This is in marked contrast with the 86% inhibition effected in untreated cells (Fig. 3B). Furthermore, the currents recorded with $3 \mu\text{M}$ ω -CTx-GVIA in the pipette from protease-treated

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slices (Fig. 5B) were larger than currents recorded with 3 μ M ω -CTx-GVIA in the pipette from untreated slices (Fig. 3B) ($P = 0.02$, Student's t -test). The attenuation of the sensitivity of the currents to ω -CTx-GVIA by prior treatment of the cells with protease was not due a general alteration of the channel-pharmacology, because currents in cells from the same protease-treated slices were reduced by 30 nM ω -Aga-IVA by 84% (Fig. 5B), a level of inhibition not less than the 78% inhibition in untreated cells (Fig. 3C), while currents recorded with 30 nM ω -Aga-IVA in the pipette from untreated (Fig. 3C) and protease-treated (Fig. 5B) slices did not differ in amplitude ($P = 0.36$, Student's t -test).

Discussion

The peptide toxin ω -CTx-GVIA is used as a blocker of N-type Ca^{2+} channels to determine the functional expression and physiological roles of N-type channels in neurons. Here we show that concentrations frequently used in such experiments can also inhibit P-type Ca^{2+} channels but the inhibition can be abolished if neurons are exposed to the enzyme protease type XXIII.

It is well established that submicromolar concentrations of ω -CTx-GVIA maximally inhibit currents carried by low concentrations of divalent cations (≤ 5 mM Ba^{2+} or Ca^{2+}) through native N-type channels (Boland et al., 1994) and recombinant N-type channels containing a $\text{Ca}_v2.2$ subunit (Bleakman et al., 1995; Lin et al., 1997). Higher concentrations are required for block in the presence of higher concentrations of divalent cations, such as the 90-110 mM Ba^{2+} used to record single channel currents (McCleskey et al., 1987; Aosaki and Kasai, 1989; Boland et al., 1994; Feng et al., 2003). However, because the rate of block speeds up with concentration (Aosaki and Kasai, 1989; Boland et al., 1994; Bleakman et al., 1995; Feng et al., 2003), micromolar concentrations (1-3 μM) are commonly used to ensure rapid and complete block of Ca^{2+} channel currents or synaptic transmission investigated in the presence of relatively low concentrations of divalent cations (e.g. McDonough et al., 1996; Sidach and Mintz, 2000). Our finding that 3 μM ω -CTx-GVIA inhibits the current carried by 5 mM Ba^{2+} in mature and immature Purkinje cells by 66%, in which most of the current is also inhibited by P-type-selective concentrations of ω -Aga-IVA, demonstrates that ω -CTx-GVIA also inhibits P-type channels. This suggests that a current component ascribed to N-type channels on the basis of inhibition by micromolar concentrations of ω -CTx-GVIA may be overestimated. The apparent reversibility of the inhibition of P-type channels contrasts with the irreversible inhibition of N-

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type channels. However, discrimination between N-type and P-type channels on this basis may be difficult since ω -CTx-GVIA may not readily washout from brain slices. Local application from a micropipette allows reversibility to be tested, since it limits exposure of the drug to non-specific binding sites, but the rate of block is relatively slow and the effective concentration at the cell of interest is much lower than in the micropipette (Tringham et al., 2007).

Inhibition of somatic P-type currents in Purkinje cells by ω -CTx-GVIA, at concentrations above those that block N-type currents ($\geq 1 \mu\text{M}$), is the converse of the P-type-versus-N-type selectivity of ω -Aga-IVA, which inhibits N-type currents at concentrations above those that block P-type currents ($\geq 1 \mu\text{M}$) (Sidach and Mintz, 2000). Inhibition of P-type currents by ω -CTx-GVIA has not been reported previously, but is predicted to occur by binding studies in which micromolar concentrations of ω -CTx-GVIA displaced binding of ω -CTx-MVIIC from P-type channels in rat brain membranes whereas subnanomolar concentrations displaced ω -CTx-GVIA from N-type channels (Lewis et al., 2000). Also, there is a high percentage of amino acid homology between the $\text{Ca}_v2.2$ and $\text{Ca}_v2.1$ proteins that form the core of native N-type and P-type channels (up to 65% overall and 78-89% within transmembrane regions). Conversion of the amino acids in the III S5-S6 linker of $\text{Ca}_v2.2$ implicated in the binding of ω -CTx-GVIA to N-type channels, to the corresponding residues in $\text{Ca}_v2.1$ III S5-S6, slowed the rate of block 21-30-fold but did not abolish it (Ellinor et al., 1994; Feng et al., 2003). Furthermore, DRG neurons, PC12 cells, sympathetic and neostriatal neurons express a Ca^{2+} channel current component that is reversibly inhibited by ω -CTx-GVIA, in addition to the predominant irreversibly-inhibited N-type current (Aosaki and Kasai, 1989; Plummer et al., 1989; Mermelstein and Surmeier, 1997). It appears not to be carried by L-type channels (Plummer et al., 1989; Mermelstein and Surmeier, 1997), but it

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is unclear if P-type channels contribute to this current since the toxins ω -Aga-IVA and ω -CTx-MVIIC were not widely available at the time of many of these studies. Nevertheless, in view of our finding that protease XXIII abolishes inhibition of P-type channels in young Purkinje cells by ω -CTx-GVIA, it is intriguing that in these studies neurons were dissociated by a non-enzymatic method or by use of an enzyme other than protease XXIII.

At odds with our finding that ω -CTx-GVIA inhibits P-type currents in Purkinje cells is the lack of effect of ω -CTx-GVIA on currents through expressed cloned $\text{Ca}_v2.1$ subunits (Mori et al., 1991; Stea et al., 1994; Ellinor et al., 1994). The use of a high concentration of Ba^{2+} (40 mM) as a charge carrier may have prevented an effect of ω -CTx-GVIA on $\text{Ca}_v2.1$ (Aosaki and Kasai, 1989; Boland et al., 1994) in some (Mori et al., 1991; Stea et al., 1994) but not in all studies (Ellinor et al., 1994). Alternatively, the different sensitivities to ω -CTx-GVIA could reflect expression of variants of $\text{Ca}_v2.1$ and/or accessory subunits that are not the same as those in rat cerebellar Purkinje cells. Rat cerebellar Purkinje neurons express multiple $\text{Ca}_v2.1$ mRNAs (Kanumilli et al., 2006) whereas the $\text{Ca}_v2.1$ clones used in two of the heterologous expression studies were isolated from rabbit brain and show splicing patterns that are not the same as those of $\text{Ca}_v2.1$ mRNAs in rat Purkinje cells (Mori et al., 1991; Ellinor et al., 1994). Purkinje neurons also express the accessory Ca^{2+} channel subunits $\alpha_2\delta_2$ and β_2 , β_3 , β_4 , whereas the rabbit $\text{Ca}_v2.1$ clones were co-expressed with $\alpha_2\delta_1$ and β_1 cloned from skeletal muscle (Mori et al., 1991; Ellinor et al., 1994). Other studies of cloned P-type channels, using other $\text{Ca}_v2.1$ variants and other accessory subunits, do not appear to have tested the effects of ω -CTx-GVIA.

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The protease-induced removal of block of Ca^{2+} channel currents in immature Purkinje cells by ω -CTx-GVIA but not by ω -Aga-IVA suggests a selective proteolytic action at a site(s) on P-type channels with which ω -CTx-GVIA interacts but not at the IVS3-4 extracellular loop of $\text{Ca}_v2.1$, which helps determine the potency of block by ω -Aga-IVA (Bourinet et al., 1999; Hans et al., 1999). This finding, and the fact that irreversible block of N-type channels in sympathetic neurons is made reversible if dissociation of sympathetic neurons cells is performed with collagenase and protease XXIII rather than with collagenase and dispase (Boland et al., 1994), suggests that the site(s) with which ω -CTx-GVIA interact(s) may be particularly susceptible to proteolysis by protease. The implication is that Ca^{2+} channel pharmacology in cells isolated from young animals and dissociated with protease XXIII may not be comparable with that of Ca^{2+} channels in brain slices or of cloned Ca^{2+} channels expressed in cell lines or *Xenopus* oocytes. We cannot be certain that protease has the same effect on Ca^{2+} channel currents in mature Purkinje cells. Our attempts to record from mature Purkinje cells after exposure to protease XXIII did not yield recordings of sufficient quality to allow the effect of ω -CTx-GVIA to be examined.

Although we have referred to the ω -Aga-IVA-sensitive channels as P-type channels containing a $\text{Ca}_v2.1$ subunit, in accordance with a large body of literature, we cannot exclude a minor contribution from channels containing a $\text{Ca}_v2.3$ subunit. $\text{Ca}_v2.3$ protein and mRNA are expressed in Purkinje cells but unlike Ca^{2+} channel currents in mature and immature Purkinje cells, currents through cloned channels containing $\text{Ca}_v2.3$ are not blocked by ω -CTx-MVIIC and there are conflicting reports of their sensitivity to ω -Aga-IVA (for references, see Tringham et al., 2007).

Inhibition of P-type currents in Purkinje cells by ω -CTx-GVIA implies that the currents may also be inhibited by the closely-related ω -CTx-MVIIA (ziconotide). This shows a weaker selectivity than ω -CTx-GVIA for brain N-type over P-type channels in binding studies (Lewis et al. 2000). It partially inhibits currents through cloned channels containing $\text{Ca}_v2.1$ at micromolar concentrations (3 μM , 20%) but completely inhibits $\text{Ca}_v2.2$ currents at 300 nM (Bleakman et al., 1995; Feng et al., 2003). Our preliminary experiments (Payne and Usowicz, unpublished) suggest that ω -CTx-MVIIA also inhibits P-type currents in Purkinje cells. Inhibition of P-type channels could help explain some of the side-effects associated with the use of ω -CTx-MVIIA in the treatment of chronic pain (Snutch, 2005; McGivern, 2006), which cannot be explained by its primary mechanism of action, inhibition of N-type channels.

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Footnotes.

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Name and address for correspondence: Dr Maria M. Usowicz, Dept. of Pharmacology, University of Bristol, University Walk, Bristol BS8 1TD, UK. Tel: 44(0)117-331-1457. Fax: 44(0)117-925-0168. E-mail: m.m.usowicz@bris.ac.uk

EWT, present address, NeuroMed Pharmaceuticals Inc., 301-2389 Health Sciences Mall, Vancouver, British Columbia, V6T 1Z4, Canada.

CEP, present address, Pfizer Global Research and Development, Sandwich Laboratories, Ramsgate Road, Sandwich, Kent CT13 9NJ, UK.

Legends for Figures

Figure 1. Ca^{2+} channel currents in somatic patches of mature cerebellar Purkinje cells (P40-50) are reduced by ω -CTx-GVIA. A-C, superimposed Ca^{2+} channel currents (charge carrier: 5 mM Ba^{2+}) recorded in cell-attached patches with a drug-free pipette (A, 74 patches) and with pipettes containing 3 μM ω -CTx-GVIA (B, 25 patches) or 20-30 μM ω -CTx-GVIA (C, 9 patches). In this and subsequent figures, each current trace is the mean of currents evoked by 15-30 depolarising voltage ramps (A, upper line), from which a linear leak current has been subtracted (see methods). D, concentration-dependence of inhibition of the peak current, I_{pk} , by ω -CTx-GVIA. Symbols and vertical bars plot mean $\pm$ S.E.M. of measurements of I_{pk} in the number of patches given in parentheses. The solid curve is the best fit with the logistic equation, for which $IC_{50} = 1.6 \mu\text{M}$, Hill slope = 2.27, max-min = 28.61 pA (80%). The fit was weighted by the inverse of the square of the S.E.M. at each point. Nevertheless, the IC_{50} and Hill slope values should be considered approximate, given the high S.E.M. and low n values at 100 nM and 1 μM . E, bar chart showing that the mean I_{pk} recorded from somata that were previously exposed to ω -CTx-GVIA from a micropipette (20 μM) and then 'washed', is not different from the mean I_{pk} recorded from somata exposed to drug-free solution from a micropipette. F, bar chart comparing the control mean I_{pk} with the mean I_{pk} recorded in the presence of 30 nM ω -Aga-IVA ($P < 0.05$, ANOVA), 30 nM ω -Aga-IVA plus 100 nM ω -contoxin GVIA ($P > 0.05$, ANOVA), and 30 nM ω -Aga-IVA plus 30 μM ω -CTx-GVIA ($P < 0.05$, ANOVA).

Figure 2. Whole-cell Ca^{2+} channel currents in mature cerebellar Purkinje cells are slowly inhibited during superfusion of ω -CTx-GVIA on to the cerebellar slice. Plot of the peak of the whole cell current, I_{pk} , evoked by voltage ramps applied at 0.05 Hz, against time, recorded in the

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absence of drug (empty circles, mean of thirteen cells; vertical bars, $\pm$ S.E.M) and during perfusion of 3 μ M ω -CTx-GVIA from a 360 μ m needle positioned $\sim$ 0.5 cm from the cell soma (filled circles and vertical bars, mean $\pm$ S.E.M of four cells). Currents were normalized by size at time zero. Charge carrier: 2 mM Ba²⁺. The decrease in I_{pk} was not accompanied by a change in holding current (zero current level is represented by the dashed line for both traces). I_{pk} was measured from the leak current (dotted line), which was estimated by fitting a straight line to the linear current recorded during the negative potentials of the voltage ramp ($\sim$ -85 mV to $\sim$ -55 mV) that did not evoke an inward current and avoided the capacitative current generated at the start of the ramp. The superimposed traces in the inset are example whole-cell currents recorded from a single Purkinje cell before and during application of ω -CTx-GVIA. Cell soma capacitance, 20 pF; current density, 213 pA pF⁻¹, control; 159 pA pF⁻¹, ω -CTx-GVIA.

Figure 3. Ca²⁺ channel currents in somatic patches of young cerebellar Purkinje cells (P13-20) are reduced by ω -CTx-GVIA. A, superimposed Ca²⁺ channel currents (5 mM Ba²⁺) recorded in somatic cell-attached patches in response to depolarising voltage ramps (top) with drug free pipettes (22 patches) and with pipettes containing 3 μ M ω -CTx-GVIA (9 patches) or 20 μ M ω -CTx-GVIA (8 patches). B, bar chart summarising the mean I_{pk} ($\pm$ S.E.M., number of patches in parentheses) recorded with pipettes containing the concentrations of ω -CTx-GVIA (3-20 μ M) that produced maximal or near-maximal block in mature cells (Fig. 1). C, bar chart confirming that 30 nM ω -Aga-IVA inhibited most of the current ($P = 0.04$, Student's t -test). D, bar chart showing that the mean I_{pk} of currents recorded in somatic patches is not altered by the prior application of ω -CTx-GVIA to the soma from a micropipette (20 μ M).

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Figure 4. Whole-cell Ca^{2+} channel currents in immature Purkinje cells are inhibited by local application of ω -CTx-GVIA to the soma from a micropipette. A, plot of whole-cell I_{pk} normalized to I_{pk} at time zero, against time for recordings in which a micropipette (pipette symbol) was used to apply drug-free extracellular solution to the soma (empty circles and vertical bars, mean $\pm$ S.E.M., five cells) or solution containing 20 μM ω -CTx-GVIA (filled circles, eight cells). Time zero denotes the start of the application. Charge carrier: 2 mM Ba^{2+} . B, time-course of whole-cell I_{pk} in a single cell (filled circles) demonstrating reversibility of the inhibition by ω -CTx-GVIA, after the micropipette was removed from the vicinity of the soma and the slice was superfused with standard drug-free solution. The recovery from inhibition by ω -CTx-GVIA was complete, if rundown is taken in to account by comparing the current size with the time-course of the mean whole-cell I_{pk} in the absence of drug (empty circles, same data as in A). The inset shows superimposed current traces recorded from a single cell before, during and after application of ω -CTx-GVIA. Cell soma capacitance, 27 pF; current density: 210 pA pF^{-1} , control, 167 pA pF^{-1} , ω -CTx-GVIA.

Figure 5. Ca^{2+} channel currents in the soma of young cerebellar Purkinje cells treated with protease are not inhibited by ω -CTx-GVIA. A, superimposed traces of Ca^{2+} channel currents evoked by depolarizing voltage ramps in cell-attached patches of cells that had been exposed to protease XXIII for ~ 7 minutes. B, bar chart showing that the mean I_{pk} of currents recorded from protease-treated cells was not significantly smaller than in untreated cells ($P = 0.53$, Student's t-test), and the mean I_{pk} in protease-treated cells was not reduced by 3 μM ω -CTx-GVIA ($P > 0.05$, ANOVA) but was reduced by 30 nM ω -Aga-IVA ($P < 0.05$, ANOVA).

Figure 1

Figure 2

P13-20

Figure 3

Figure 4

Figure 5