J. Physiol. (1987), **383**, *pp.* 487–498
With 8 text-figures
Printed in Great Britain

α-ADRENERGIC INHIBITION OF RAT CEREBELLAR PURKINJE CELLS *IN VITRO*: A VOLTAGE-CLAMP STUDY

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(*Received 21 April 1986*)

SUMMARY

1. The effects of the α_2-adrenergic agonist clonidine on the membrane properties of Purkinje cells were analysed in sagittal slices of adult rat cerebellum by the use of intracellular recordings performed at a somatic level in the single-electrode voltage-clamp mode.

2. In preliminary current-clamp experiments, clonidine elicited in all cells a hyperpolarization 3–8 mV in amplitude, accompanied by a 15–35% increase of the input resistance when it was added to the bath at a concentration of 2–5 μM.

3. In voltage-clamped cells at a potential of −65 mV. the same concentration of clonidine always induced an outward shift of the holding current (0·2–0·5 nA in amplitude), thus corresponding to the hyperpolarization seen in current-clamp experiments, and this effect was accompanied by a clear increase of membrane resistance. Furthermore, clonidine markedly depressed the inward relaxations induced by hyperpolarizing commands of amplitude less than 10–20 mV whereas those induced by larger steps were much less affected. All these effects of clonidine were reversible when the drug was washed out.

4. When the slices were bathed in a medium containing 10 mM-Cs and 5×10^{-6} M-tetrodotoxin, the inward relaxations induced by hyperpolarizing steps were abolished. However, a small inward current was still present when the membrane potential was stepped back to −65 mV, which was in turn blocked by the Ca-channel blocker Cd. This inward Ca current was also blocked by 2–5 μM-clonidine in the bath.

5. All these effects of clonidine were abolished by the α_1-adrenergic antagonists prazozin and phentolamine at concentrations of 0·5 and 40 μM respectively in the bath. In contrast, they were only weakly antagonized or unaffected by 2 μM of the α_2-adrenergic antagonist yohimbine.

6. On the basis of these results and of a previous work on the ionic basis of the inward rectification of Purkinje cells (Crepel & Penit-Soria, 1986), it appears that these neurones exhibit a well developed α (possibly α_1)-adrenergic inhibition of a low-threshold Ca conductance and a Ca-dependent K conductance operating near resting potential.

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INTRODUCTION

The cerebellum of mammals receives a rich noradrenergic innervation originating in the locus coeruleus (Bloom, 1978). In the molecular layer of the cerebellar cortex, noradrenergic fibres make synapse-like contacts with secondary or tertiary Purkinje cell dendrites (Ito, 1984). In a series of *in vivo* experiments, it was shown that noradrenaline hyperpolarized and increased the input resistance of Purkinje cells and suggested that these effects are probably mediated through cyclic adenosine monophosphate (see Bloom, 1978). In more recent experiments on cerebellar slices *in vitro*, Basile & Dunwiddie (1984) showed noradrenaline to have both excitatory and inhibitory effects on the extracellularly recorded spontaneous firing of Purkinje cells, depending on the concentration of the amine in the bath. Furthermore, excitatory effects were mimicked by the β-adrenergic agonist isoprenaline whereas the inhibitory ones were mimicked by the α_2-adrenergic agonist clonidine.

In the present series of experiments, the effects of clonidine on Purkinje cells in cerebellar slices *in vitro* were further analysed, with special emphasis on the possible action of this drug on voltage-dependent conductances operating near resting potential, namely on the inward rectifier I_h, the Ca-dependent K conductance ($g_{K,Ca}$) and the low-threshold Ca conductance previously identified in these neurones (Crepel & Penit-Soria, 1986). A preliminary account of this work has been published previously (Crepel, Debono & Flores, 1986).

METHODS

Experiments were performed on sagittal (400 μm thick) cerebellar slices of male Wistar rats (1 or 2 months old) maintained *in vitro*. The methods used for preparing the slices, the recording chamber, and the composition of the standard and modified bathing solutions were the same as described previously (Crepel, Dahnjal & Garthwaite, 1981; Crepel & Penit-Soria, 1986). The bathing medium was continuously gassed with a mixture of O_2 (95 %) and CO_2 (5 %) and maintained at 35 °C. Under direct visualization of the cortical layers, Purkinje cells were voltage-clamped at a somatic level by using a Wilson–Goldner switch-clamp circuit (Wilson & Goldner, 1975) (Dagan 8100). Glass micro-electrodes were filled with 3 M-KCl (40–70 MΩ). The procedure used to clamp the cells in the single-electrode voltage-clamp mode was the same as that described previously (Galvan & Adams, 1982; Halliwell & Adams, 1982; Crepel & Penit-Soria, 1986). In the present experiments the switching frequency was 3 kHz with a duty cycle of 25 %, and the maximum gain which could be achieved was 2000–3500, depending on the electrical properties of the micro-electrodes. The holding potential of the cells was maintained at −65 mV, i.e. below the firing level for Na and Ca spikes (Llinás & Sugimori, 1980*a*, *b*). As previously stated (Crepel & Penit-Soria, 1986), cells under such conditions were not perfectly clamped, probably because the gain was still lower than the value required to ensure an adequate voltage clamp. The cable properties of Purkinje cells prevented them from being isopotential during the 30–50 ms which followed a voltage step, as already shown by Johnston & Brown (1983) for hippocampal neurones. However, the time course of the current relaxations studied was much longer, so that they were not grossly distorted by the limitations of the voltage clamp (Crepel & Penit-Soria, 1986). Analysis of the data was performed on a Goupil 3 microcomputer, with a sampling frequency of 1 kHz. Data were also stored on tape for further analysis (5 kHz band width).

RESULTS

In a preliminary series of experiments, the effects of clonidine on Purkinje cells were analysed in the current-clamp mode. In the eight cells tested, a hyperpolarization of 3–8 mV was observed when 2–5 μM-clonidine was added to the bath. This effect was accompanied by a 15–35% increase of the input resistance. Full recovery was observed when clonidine was washed out (Fig. 1).

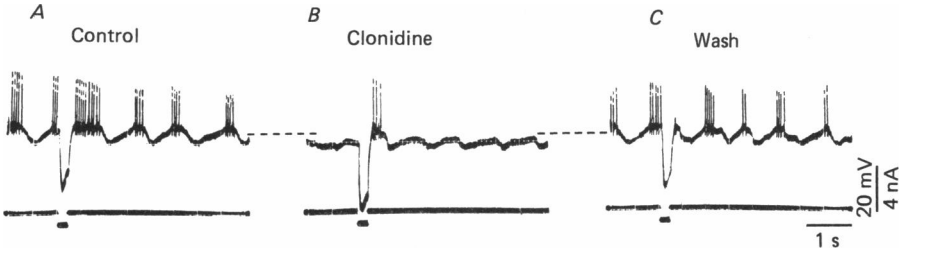

Fig. 1. Effects of clonidine on membrane potential and input resistance of Purkinje cells. *A*, control. The downward deflexion in the voltage record (upper trace) is the hyperpolarizing response of the cell to a current pulse of 1 nA (lower trace) injected through the recording micro-electrode. Note the oscillatory behaviour of the membrane potential. *B*, same cell as in trace 1 after the addition of 5 μM-clondidine in the bath, which induced a hyperpolarization of about 5 mV accompanied by an increase in the amplitude of the response to the same injection of current. Note the presence of a depolarizing response at the end of the hyperpolarizing pulse, giving rise to a train of fast spikes. *C*, recovery after washing out of the drug. The spikes are truncated by the frequency response of the recording system and retouched in all traces.

These results are in complete agreement with those of Basile & Dunwiddie (1984) on the inhibitory effect of this drug on the spontaneous firing of Purkinje cells.

In the following sections, the cells were studied in the single-electrode voltage-clamp mode (see Methods).

Effects of clonidine on the inward rectification of Purkinje cells

In the thirty-eight successfully voltage-clamped Purkinje cells (mean resting potential $= -60 \pm 0{\cdot}5$ mV; mean input resistance $= 19 \pm 1{\cdot}6$ MΩ), jumps from the holding potential of −65 mV to more negative potentials always elicited well developed time- and voltage-dependent inward relaxations ('on' relaxation) following the initial transients (Fig. 2*A*1). As shown previously (Crepel & Penit-Soria, 1986), the 'on' relaxations were monotonic in most Purkinje cells, which allowed them to be extrapolated back to time zero to determine the instantaneous current values. This in turn allowed the construction of instantaneous and steady-state *I–V* curves for each neurone (Fig. 2*A*2). These, as well as the direct examination of the original records, showed that the inward rectification was already present near −65 mV in all cells and increased to reach values of 0·8–3 nA, depending on the cells, for jumps up to about −120 mV.

When clonidine was added to the bath at a concentration of 2–5 μM, three effects were observed. First, there was a 0·2–0·5 outward shift of the holding current in all

cells ($n = 9$). Secondly, the inward relaxations induced by hyperpolarizing commands of amplitude less than 10–20 mV were depressed or abolished, whereas those induced by larger hyperpolarizing steps were left almost unaffected (Fig. 2*B*1 and 2). Thirdly, the membrane resistance of the cells was clearly increased, as revealed by the decrease

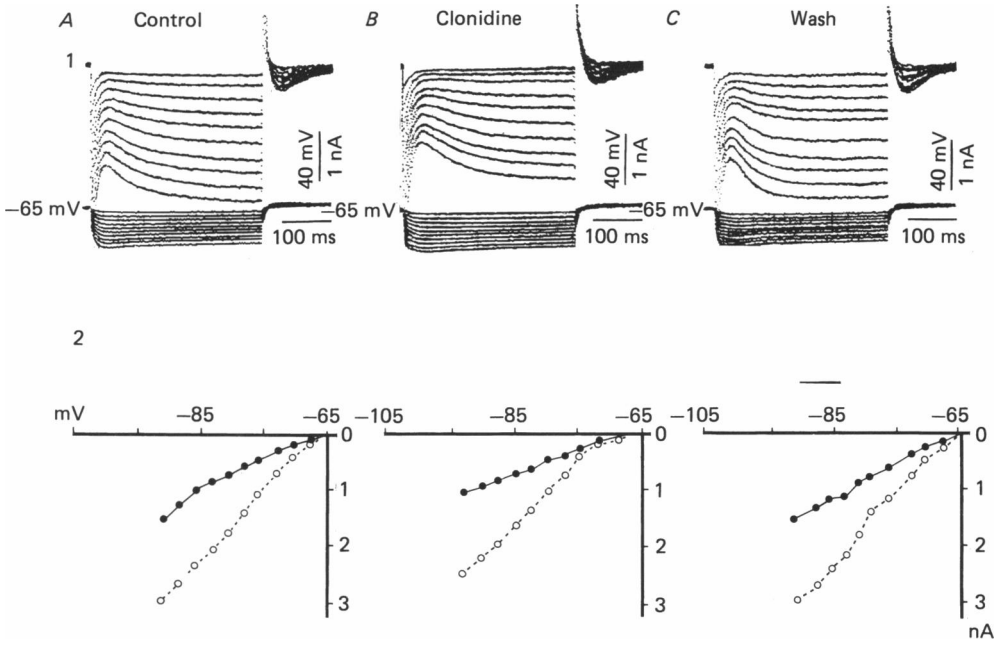

Fig. 2. Effects of clonidine on the inward rectification. *A*1, current relaxations (upper traces) induced by the voltage commands shown in the lower traces in standard Ringer solution. Holding potential −65 mV (full explanation in text). *A*2 *I–V* relationships of the 'on' relaxations of this neurone. ●—●, instantaneous *I–V* plots (full explanation in text); ○--○, steady-state *I–V* relationships. *B*1 and 2, same as in *A*1 and 2 in the same cell in the presence of 5 μM-clonidine in the bath. Note that the 'on' relaxations were markedly reduced near resting potiential and that the slope of the instantaneous *I–V* curve was smaller. *C*1 and 2, recovery after washing out of clonidine. Each trace is the average of ten traces.

of the slope of the instantaneous *I–V* curves (compare *A*2 and *B*2 in Fig. 2). All these effects were reversible when clonidine was washed out (Fig. 2*C*1 and 2).

In a previous series of experiments (Crepel & Penit-Soria, 1986) we showed that the inward relaxations near resting potential, i.e. between −65 and −75 to −85 mV are probably due to the closure of a $g_{K,Ca}$ which is open at rest, whereas at more negative membrane potentials they are due to the turning on of the inward rectifier I_h (Bader & Bertrand, 1984). Accordingly, it is likely that clonidine markedly depresses $g_{K,Ca}$ operating near rest whereas it has little or no effect on I_h. However, in this case one might expect the holding current to shift inwards rather than outwards as observed (see above). One possible explanation of this apparent discrepancy might be that clonidine also depresses an inward current present near the resting potential. A good candidate might be the low-threshold Ca current which participates in anodal breaks in Purkinje cells (Crepal & Penit-Soria, 1986). Since the

presence of this current was only demonstrated by conventional intracellular recordings in these experiments, we decided to carry out a voltage-clamp study of this low-threshold Ca currrent and of the effects of clonidine on this conductance.

Low-threshold Ca conductance in Purkinje cells

In standard Ringer solution, 'on' relaxations induced by hyperpolarizing voltage jumps from a holding potential of −65 to −100 mV were followed by well developed 'off' relaxations when the membrane potential was stepped back to −65 mV (Figs. 2 and 3*A*). When the inward relaxation was blocked by adding 10 mM-Cs to the

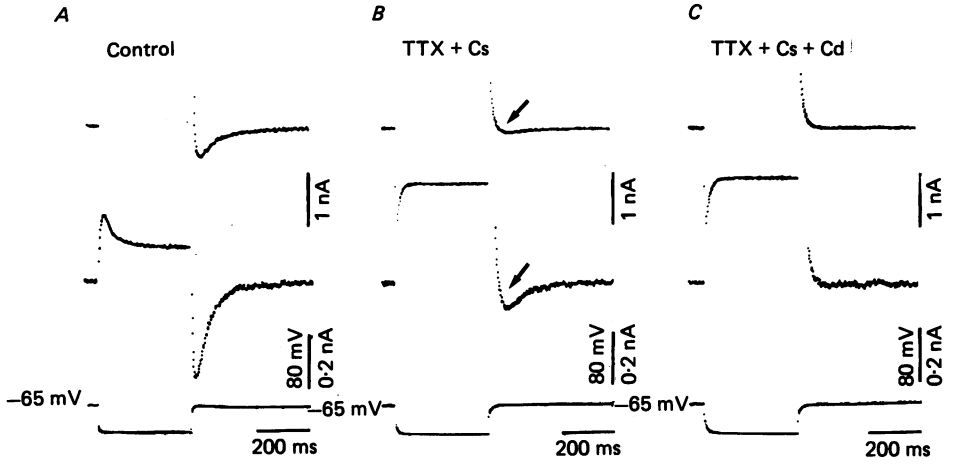

Fig. 3. Low-threshold Ca conductance in Purkinje cells. *A*, current relaxation (low-gain and high-gain records in the upper trace and in the middle trace respectively) induced in a cell by a 40 mV hyperpolarizing command (lower trace) in standard Ringer solution. *B*, same as in *A* following the addition of 5×10^{-6} M-TTX and 10 mM-Cs to the bath. Note the disappearance of the 'on' relaxation and the presence of a small inward current (arrow) when the membrane potential was stepped back to −65 mV. In *C*, this inward current was in turn abolished when Ca was replaced by 1 mM-Cd in the bath. Each trace is the average of fifty sweeps.

bathing medium (Fig. 2*B*) and when possible complexities due to the slow Na current activated near resting potential (Llinás & Sugimori, 1980*a*; Crepel & Penit-Soria, 1986) were eliminated by $5–10^{-6}$ M-tetrodotoxin (TTX) in the bath, the disappearance of the tail currents of I_h uncovered a small inward current, 0·10–0·25 nA in amplitude (Fig. 3*B*).

In the four cells tested, this inward current was in turn blocked when Ca was replaced by the Ca-channel blocker Cd (1 mM) in the bath (Fig. 3*C*), indicating that Ca ions were its main charge carrier. Therefore, the present results fully corroborate the previous assumption (Crepel & Penit-Soria, 1986) that Purkinje cells exhibit a low-threshold Ca conductance which can be activated near the resting potential by a hyperpolarizing voltage jump. Furthermore, in the presence of Cs and TTX, there was always a small (0·05–0·2 nA) outward shift of the holding current when Ca was replaced by Cd, suggesting that a fraction of these Ca channels are open at rest. Finally, in this latter bathing medium (Cs+TTX+Cd), the responses of Purkinje

cells to hyperpolarizing voltage jumps appeared to be purely passive (Figs. 3*C* and 4). Indeed, subtracting these responses from those elicited in standard Ringer solution by the same voltage jump completely cancelled the transients of the response in one of the four cells tested, thus revealing the full time course of the 'on' relaxation due to I_h. As expected for this current, the 'on' relaxation started at the very beginning

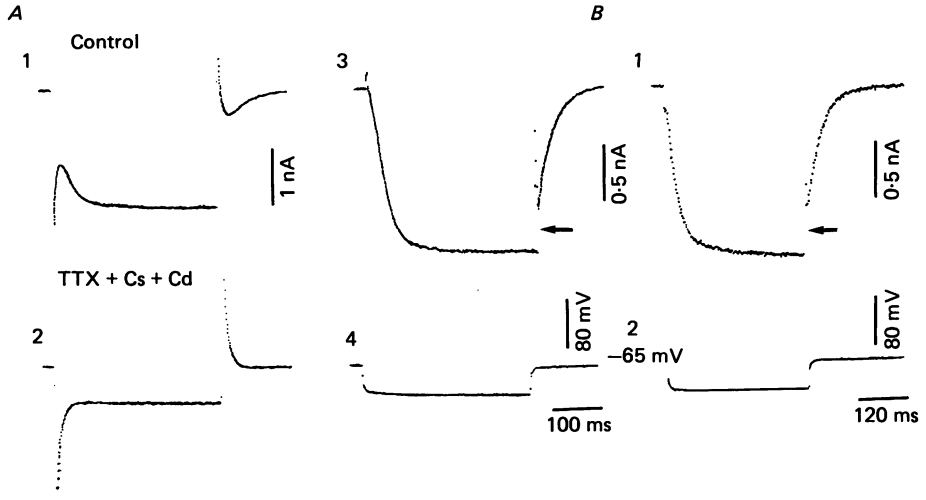

Fig. 4. Time course of the inward rectification. *A*1, current relaxation induced in a Purkinje cell by the voltage step shown in *A*4 in standard Ringer solution. *A*2, same as in *A*1 in the presence of 5×10^{-6} M-TTX, 10 mM-Cs and 1 mM-Cd in the bath. *A*3, time course of the 'on' and 'off' relaxations of this cell obtained by subracting the trace shown in *A*2 from that illustrated in *A*1. The arrow indicates the ohmic step present at the end of the voltage step. *B*1 and 2, same as in *A*3 and 4 in another cell. Each trace is the average of fifty sweeps.

of the voltage jump and an ohmic step was present at the end of the pulse, due to the membrane resistance decrease during the turning on of I_h (Fig. 4*A*). In the three other cells similar results were obtained, except that a small ohmic current was present at the beginning of the voltage jump (Fig. 4*B*), probably due to the fact that the membrane resistance of the cells often increased in Cd-containing medium (Crepel & Penit-Soria, 1986).

Effect of clonidine on the low-threshold Ca conductance

In the seven cells tested, the small inward current elicited at the end of a voltage jump from −65 to −100 mV in a bathing medium containing 10 mM-Cs and 5×10^{-6} M-TTX was completely blocked by 2–5 μM-clonidine in the bath (Fig. 5). This effect was accompanied by a 0·2–0·5 nA outward shift of the holding current and also by a small decrease in membrane conductance (Fig. 5).

From these results, it can be concluded that clonidine markedly depresses the low-threshold Ca conductance of Purkinje cells.

Effects of α-adrenergic antagonists

In the present series of experiments, the concentrations of clonidine in the bath were similar to those previously used by Basile & Dunwiddie (1984), i.e. rather high

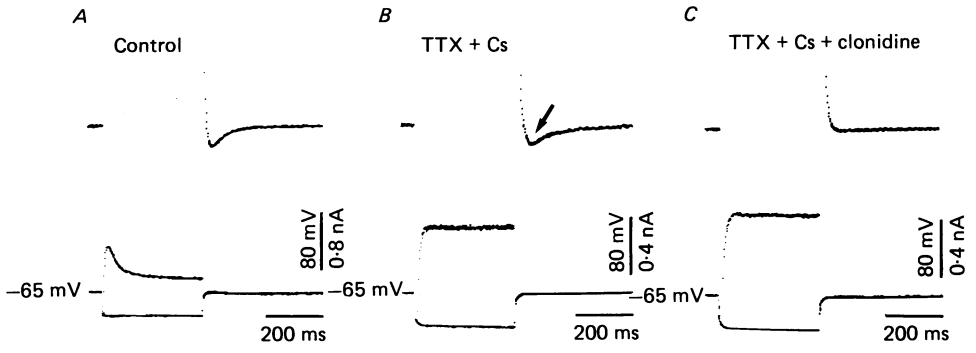

Fig. 5. Effect of clonidine on the low-threshold Ca current. *A*, current relaxation (upper trace) induced in a cell by the hyperpolarizing command shown in the lower trace in control medium. *B*, same as in *A* following the adddition of 5×10^{-6} M-TTX and 10 mM-Cs to the bath. Note the presence of a small inward Ca current (arrow) at the end of the voltage step. *C*, this current was abolished by the addition of 2 μM-clonidine to the bath. Each trace is the average of fifty sweeps.

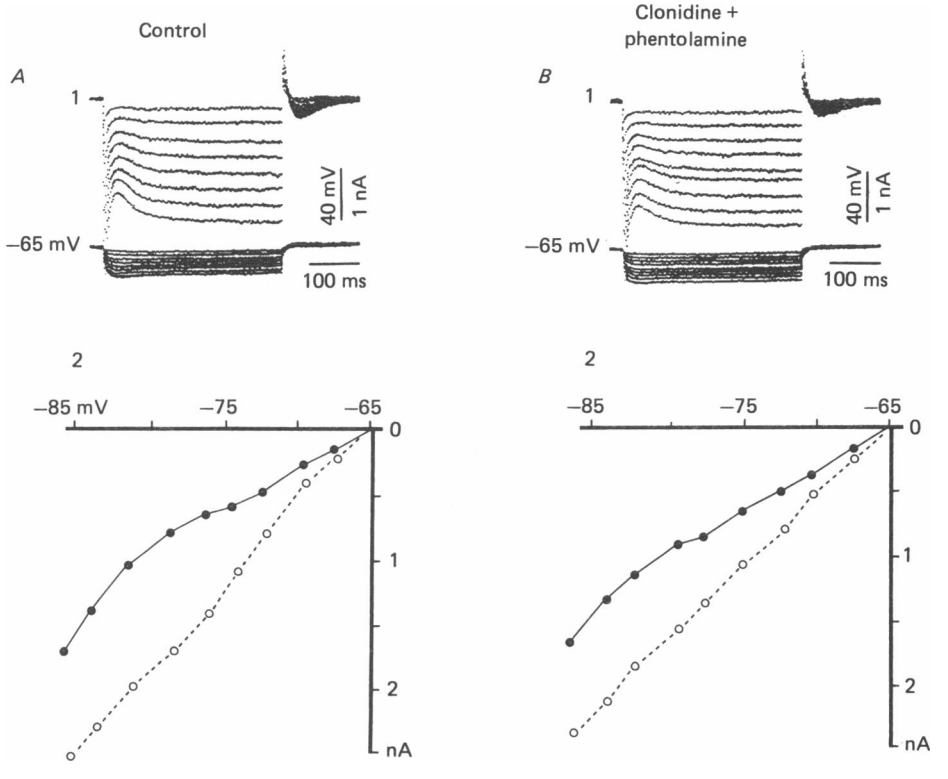

Fig. 6. Lack of effect of clonidine on the inward rectification in the presence of phentolamine. *A*1, control responses to various current pulses in a Purkinje cell in standard Ringer solution. *A*2, *I–V* relationship for this cell. *B*1 and 2, same as in *A*1 and 2 following the addition of 2 μM-clonidine and 40 μM-phentolamine to the bath. In *A*1 and *B*1, each trace is the average of ten sweeps. ●—●, instantaneous *I–V* plots (full explanation in text); ○---○, steady-state *I–V* relationships.

with respect to concentrations known to fully activate α_2-adrenoreceptors in other preparations (Starke, Endo & Taube, 1975; Wilberg, 1978*a*, *b*; U'Prichard, Greenberg & Snyder, 1977). This raises the question of the specificity of the action of clonidine in the present study. This problem was tackled by the use of selective α-antagonists.

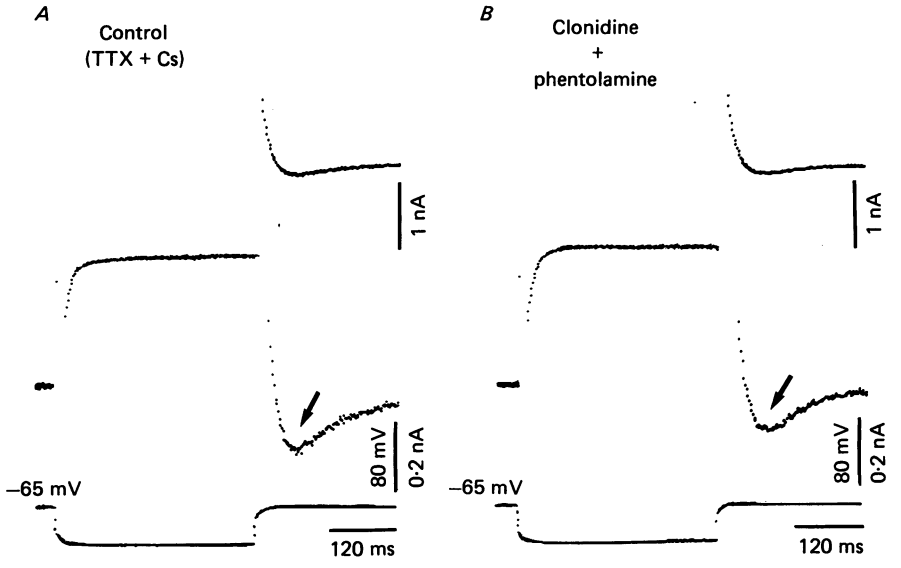

Fig. 7. Lack of effect of clonidine on the low-threshold Ca current in the presence of phentolamine. *A*, inward Ca current (low-gain and high-gain records in the upper and in the middle trace respectively) induced at the end of a hyperpolarizing command shown in the lower trace, in the presence of 5×10^{-6} M-TTX and 10 mM-Cs in the bath. *B*, same as in *A* in the presence of 5 μM-clonidine and 40 μM-phentolamine in the perfusing solution. Note the persistence of the Ca inward current (arrows in *A* and *B*). Each trace is the average of fifty sweeps.

All the effects of clonidine described so far were fully antagonized by the α-adrenergic antagonist phenotolamine (40 μM) in the bath ($n = 6$). This was true for the effects of clonidine on the inward rectification (Fig. 6*A* and *B*), on the membrane resistance (Fig. 6*A* and *B*), and on the inward Ca current elicited at the end of a hyperpolarizing voltage jump in Cs and TTX bathing medium (Fig. 7).

Prazozin, a very potent and more selective α_1-adrenergic antagonist in other tissues (references in Exton, 1985), also fully antagonized the effects of clonidine ($n = 6$) on the inward rectification, the low-threshold Ca current and the membrane resistance of the cells, at a concentration as low as 0·5 μM in the bath (not illustrated).

In marked contrast with the other α-adrenergic antagonists tested, the selective α_2-adrenergic antagonist yohimbine (references in Exton, 1985) did not antagonize or only weakly antagonized the effects of clonidine on the inward rectification, the membrane resistance (Fig. 8*A* and *B*) and the low-threshold Ca current of Purkinje cells (Fig. 8*B*) in the six cells tested, at concentrations up to 2 μM.

Therefore, on the basis of these results, it seems likely that Purkinje cells exhibit a well developed α-adrenergic (possibly α_1) inhibition of both a low-threshold Ca conductance and of a $g_{K, Ca}$ operating near the level of the resting potential (see Discussion).

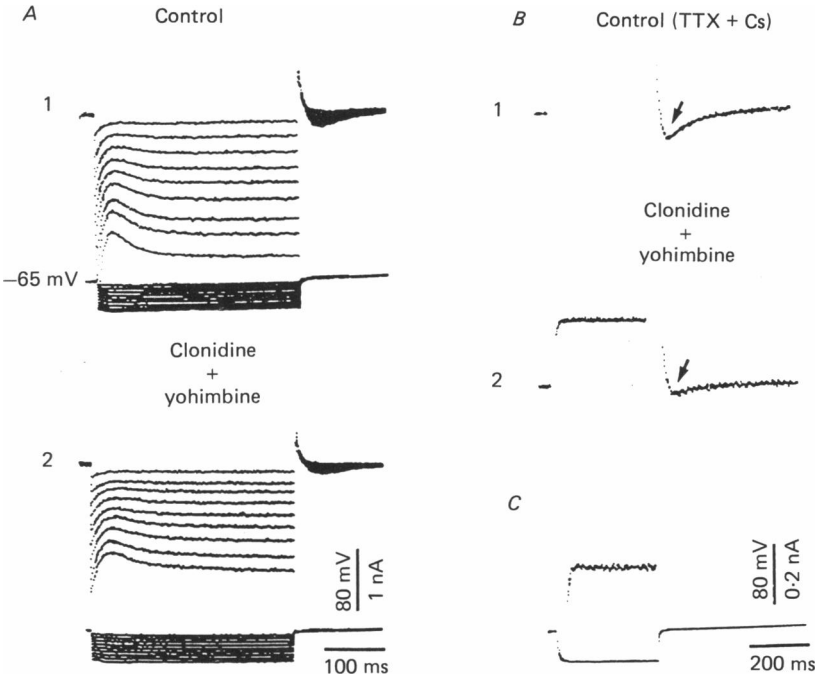

Fig. 8. Lack of antagonistic action of yohimbine on the effects of clonidine. *A*1, current relaxations (upper traces) induced by the voltage commands shown in the lower traces in standard Ringer solution. *A*2, same as in *A*1 in the presence of 5 μM-clonidine and 2 μM-yohimbine in the bath. Each trace is the average of ten sweeps in *A*1 and 2. *B*1, inward Ca current induced at the end of an hyperpolarizing command shown in *C*, in the presence of 5×10^{-6} M-TTX and 10 mM-Cs in the bath. *B*2, same as in *B*1 following the addition of 5 μM-clonidine and 2 μM-yohimbine to the perfusing solution. Note that yohimbine still markedly reduced the amplitude of the Ca current (arrows in *B*1 and 2). Each trace is the average of fifty sweeps in *B*1 and 2, and *C*.

DISCUSSION

In previous *in vitro* experiments, Basile & Dunwiddie (1984) showed clonidine to depress the spontaneous firing of Purkinje cells. The present study shows that this effect is due at least in part to a hyperpolarization of the cells, the counterpart of which is a 0·2–0·5 nA outward shift of the holding current in voltage-clamped neurones (see Results). However, because cells were held at −65 mV, i.e. at a membrane potential about 5 mV more negative than their normal resting potential, it is likely that a fraction of the outward shift of the holding current simply reflects the decrease of the membrane conductance of the cells under clonidine. In the thirty-eight cells of the present study, this holding current never exceeded 0·5 nA in standard Ringer solution. Assuming a maximum change in input resistance of 35 % under clonidine (see Results), this would lead at most to an outward shift of the holding current of less than 0·2 nA, i.e. clearly smaller than that actually observed under clonidine. From these very crude estimates it seems therefore that a sizeable fraction of the shift in the holding current under clonidine is the true outward current

underlying the hyperpolarization of the cells seen in preliminary current-clamp experiments.

The results of the present study have shown that clonidine blocks both a low-threshold Ca conductance and a $g_{K,Ca}$ open at rest. Accordingly, the outward current induced by clonidine would be in fact the sum of the inward and of the outward currents due to the closure of K and Ca channels respectively. There is no measure of the current due to $g_{K,Ca}$ at a resting potential in Purkinje cells. On the other hand, the results indicate that the amplitude of the current due to the low-threshold Ca conductance operating near rest seems rather small, i.e. ranging between 0·05 and 0·02 nA as far as can be estimated from the outward shift of the holding current induced by replacing Ca by the Ca-channel blocker Cd in a bathing medium in which $g_{K,Ca}$ had already been abolished by Cs (Crepel & Penit-Soria, 1986).

However, the majority of the Ca channels are probably located on the dendrites of Purkinje cells (Llinas & Sugimori, 1980*a*, *b*) and the cells were voltage-clamped at a somatic level, which probably introduced a bias in the measurement of the Ca currents. Nevertheless, this does not explain why the outward shift of the holding current under Cd was smaller than that induced by clonidine in the same TTX and Cs bathing medium (see Results). A possibility is that clonidine also affects other conductances which could not be measured by the present experimental arrangement. For instance, in neurones of the locus coeruleus, of the dorsal horn of the rat spinal cord and in cat parasympathetic cells, clonidine acting on α_2-adrenoceptors increases a K conductance (North & Yoshimura, 1984; Akasu, Gallagher, Nakamura, Shinnick-Gallagher & Yoshimura, 1985; Williams, Henderson & North, 1985). However, when the effects of clonidine on $g_{K,Ca}$ and on the low-threshold Ca conductance were fully antagonized by the α_1-adrenergic antagonist prazozin, we did not observe either any remaining outward shift of the holding current or any decrease in the membrane resistance under clonidine which might be attributed to the activation of α_2-adrenoceptors in Purkinje cells. Thus, the most likely interpretation of the data might be that, in addition to its blocking action on $g_{K,Ca}$ and on Ca currents, clonidine may also activate an electrogenic pump, as proposed in other cells (Lee & Phillis, 1977; Sastry & Phillis, 1977; Segal, 1981).

Further studies are required to clarify this point, as well as to determine if the effects of clonidine are direct or indirect. However, a direct action of the drug on Purkinje cell membranes is likely since in their *in vitro* experiments Basile & Dunwiddie (1984) showed that the depressing action of clonidine on the spontaneous firing of Purkinje cells was not affected in a low-Ca (1 mM), high-Mg (3·0 mM) bathing medium which strongly depressed synaptic transmission.

From the present results, it seems at first sight reasonable to conclude that the effects of clonidine on Purkinje cells are mediated through the activation of α_2-adrenoceptors in Purkinje cells. Thus, the most likely interpretation of the data antagonists prazozin and phentolamine but not by the selective α_2-adrenergic antagonist yohimbine. Indeed, in preliminary experiments (not shown), we confirmed that the β-adrenergic agonist isoprenaline has a clear excitatory effect on Purkinje cells at concentrations of 0·5–1 μM (Basile & Dunwiddie, 1984), instead of the inhibitory action observed with clonidine. Also in keeping with this conclusion, it is

noteworthy that the activation of α_2-adrenoceptors in other nerve cells leads to an hyperpolarization accompanied by an increase of membrane conductance (North & Yoshimura, 1984; Akasu *et al.* 1985; Williams *et al.* 1985) rather than by a decrease of membrane conductance as observed in Purkinje cells. However, surprisingly enough, the α_1-adrenergic agonist phenylephrine (references in Exton, 1985) only produced inconsistent effects on the spontaneous firing of extracellularly recorded Purkinje cells as well as on their membrane potential and input resistance in preliminary current-clamp experiments (not shown) at concentrations up to 10 μM in the bath. This observation supports the previous results of Basile & Dunwiddie (1984) showing that phenylephrine had no significant effect on the discharge rate of Purkinje cells at doses up to 100 μM. Therefore, the conclusion that clonidine acts on Purkinje cells through the activation of α_1-adrenoceptors will require to be confirmed by a detailed study of the effects of other selective α_1-adrenergic agonists on their membrane properties.

On the whole, the present experiments show that Purkinje cells exhibit a well developed α (possibly α_1)-adrenergic inhibition of both a $g_{K,Ca}$ and a low-threshold Ca conductance operating near resting potential. This supports previous observations indicating that noradrenaline and in particular α_1-adrenergic agonists decrease rather than increase K and Ca conductances of nerve cells (Dunlap & Fishbach, 1978; Horn & McAfee, 1980; Galvan & Adams, 1982, Haas, 1983; Aghajanian, 1985; Akasu *et al.* 1985).

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