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Potassium channel activators decrease endogenous glutamate release from rat cerebellar slices

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Summary. The effects of the sulphonylurea activators of ATP-sensitive potassium channels (K^+_{ATP}), cromakalim and pinacidil, on the evoked-release of endogenous glutamate from superfused slices of rat cerebellum was examined. K^+-stimulated release was Ca^{2+}-dependent, whereas tetrapentylammonium (TPeA)-evoked release occurred both in the presence and absence of Ca^{2+}, but was significantly greater in Ca^{2+}-free medium. The Ca^{2+}-dependent TPeA and K^+-evoked release of glutamate was inhibited by both cromakalim and pinacidil in a concentration-dependent fashion. However, although cromakalim markedly reduced Ca^{2+}-independent TPeA-evoked release, pinacidil was ineffective. In addition, the vehicle for cromakalim, ethanol, markedly potentiated both Ca^{2+}-dependent and -independent TPeA-evoked release, but not K^+-evoked release. Despite a high concentration of sulphonylurea binding sites and a dense glutamatergic innervation, the concentrations of K^+_{ATP} channel activators required to inhibit stimulus-evoked release from the cerebellum are higher than those reported to inhibit glutamate release or reduce neuronal activity in other parts of the CNS.

Keywords: Amino acids – Glutamate release – Cromakalim – Pinacidil – Cerebellar slices

Introduction

The development of a variety of specific potassium (K^+) channel modulating agents has, in recent years, attracted widespread attention. The sulphonylurea compounds in particular have merited special consideration with regard to

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possible therapeutic roles (see Cook, 1988). Cromakalim and pinacidil exert a potent relaxing effect on a variety of smooth muscle preparations (Hamilton et al., 1986; Weir and Weston, 1986 a,b; Bray et al., 1987). This relaxation is believed to occur via hyperpolarisation of the cell membrane due to efflux of K^+ through ATP-sensitive K^+ channels (K^+_{ATP} channels, Standen et al., 1989) similar to those found in pancreatic β-cells (Schmid-Antomarchi et al., 1987).

In contrast to the large number of reports on peripheral tissue preparations, less data has been published regarding the effects of K^+_{ATP} channel activators on central nervous system function, despite the identification of sulphonylurea-sensitive binding sites in the brain (Mourre et al., 1989) and the observation of behavioural effects (Tricklebank et al., 1988). Alzheimer and ten Bruggencate (1988) successfully reduced neuronal excitability with cromakalim in guinea pig hippocampal slices; intracellular recording revealed a cromakalim-induced hyperpolarization, presumably by activation of a K^+ conductance (Alzheimer et al., 1989). More recently, cromakalim has been shown to decrease the spontaneous discharge of locus coeruleus neurones (Finta et al., 1993). Gandolfo et al., (1989 a,b) reported that K^+_{ATP} channel activators prevented epileptic activity evoked by bee venom peptide and reduced seizures in genetically epileptic rats. In addition, it has been shown that hypoxia-induced depolarisation of hippocampal slices can be potentiated by K^+_{ATP} blockers and attenuated by K^+_{ATP} activators (Ben-Ari and Lazdunski, 1989; Ben-Ari et al., 1990); thus it has been suggested that K^+_{ATP} channels may serve to inhibit glutamate release in the hippocampus (Ben-Ari et al., 1989; Ben-Ari, 1990a).

The molecular layer of the cerebellar cortex contains a particularly dense innervation of glutamatergic neurones (Sandoval and Cotman, 1978) whilst also possessing the highest concentration of glibenclamide-sensitive binding sites in the cerebellum (Mourre et al., 1989). Examination of the cerebella of weaver mutant mice which lack granule cells and hence parallel fibres indicates that K^+_{ATP} channels are present on glutamatergic neurones (Mourre et al., 1990). The aim of this study was to investigate the effects of the K^+_{ATP} channel activators, cromakalim and pinacidil, on the evoked release of endogenous glutamate from rat cerebellar slices.

Material and methods

Tissue preparation

Female Wistar rats (200–250g) were killed by cervical dislocation. The brain was rapidly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF). The cerebellum was dissected and 250μm parasaggital slices were cut on a McIlwain tissue chopper. Three slices were placed on a gauze disc and transferred to the perfusion chamber. The slices were perfused at 1ml/min at 37°C, and both the chamber and ACSF were gassed with 95% O_2/5% CO_2 throughout. After 45 minutes equilibration, two-minute samples of the perfusate were collected. Full details of the method have been published elsewhere (Barnes et al., 1988).

Experimental design

The experimental procedure consisted of three two-minute pulses of elevated K^+ (35 or 60mM) or the K^+ channel blocker, tetrapentylammonium (TPeA), in a 12-minute cycle, with

the appropriate K^+-activator (or vehicle) being perfused prior to, during and following the second pulse for a total of 12 minutes. The same protocol was used when perfusing under Ca^{2+}-free conditions in the middle pulse. All results for triple pulse experiments are expressed as a percentage of glutamate released by the first pulse.

HPLC method

Reverse-phase high performance liquid chromatography with fluorometric detection was used to determine glutamate levels in the perfusate. Pre-column derivatization with o-phthaldialdehyde/2-mercaptoethanol was used to measure both the endogenous glutamate and the internal standard, homocysteic acid which was added to the samples prior to assay. A C_{18} (octadecyl) column was used for the separation and the mobile phase consisted of distilled water, sodium propionate buffer, acetonitrile, methanol and dimethylsulphoxide in the following proportions: 827 : 113 : 39 : 19 : 2.

Drugs and solutions

The following drugs were used: cromakalim (Beecham), pinacidil (Leo), TPeA (Aldrich). Stock solutions of TPeA (1×10^{-1}M), cromakalim and pinacidil (both at 1×10^{-2}M) were prepared daily, with cromakalim and pinacidil dissolved in ethanol and dimethylsulphoxide (DMSO) respectively (final concentrations in the perfusion medium were 1.4–2.8% ethanol and 2% DMSO). Control experiments were performed using both vehicles. The ACSF contained the following (mM): NaCl 124; KCl 5; NaH_2PO_4 1.25; $MgSO_4$ 2: $CaCl_2$ 2; NaH_2CO_3 26; glucose 10; pH 7.4. For elevated K^+ media (35 and 60mM) corresponding reductions in NaCl concentrations were made, and for Ca^{2+}-free medium NaCl was increased by 2mM and the Ca^{2+} chelator EGTA (1mM) added.

Statistical analysis

Statistical analysis was performed using Student's t-test for unpaired data. All data are means ± SEM. The numbers given in the legends to the figures refer to the number of animals used.

Results

Effect of elevated K^+ and TPeA on glutamate release

Endogenous glutamate release was evoked by elevated K^+ in a concentration-dependent manner which was highly Ca^{2+}-dependent indicating an exocytotic source (Barnes & Davies, 1988) but insensitive to tetrodotoxin (Dickie & Davies, 1992). The first pulse of 35 and 60mM K^+ produced approximately 6 and 9-fold increases over basal release respectively; less glutamate was released with subsequent pulses with respect to the first pulse. Basal levels of glutamate in control experiments were 18.9 ± 1.5pmol/mg tissue/2min sample ($n = 24$, mean ± sem). 35mM K^+ and 60mM K^+ (both $n = 6$) increased glutamate release to 631 ± 90 and 939 ± 157% of basal release respectively. Glutamate release to the second and third pulses of 35 and 60mM K^+ were 92 ± 5, 77 ± 7% and 88 ± 7, 72 ± 5% respectively compared to levels at the first pulse. Aspartate release was also measured, however, potassium-evoked release was small and was only elicited by the first K^+ pulse. Under normal conditions (NACSF i.e. 2mM Ca^{2+}), TPeA at a concentrations of 1mM was shown to evoke glutamate release (143 ± 6% over basal, $P < 0.05$, Fig. 1a), confirming previous data from this laboratory

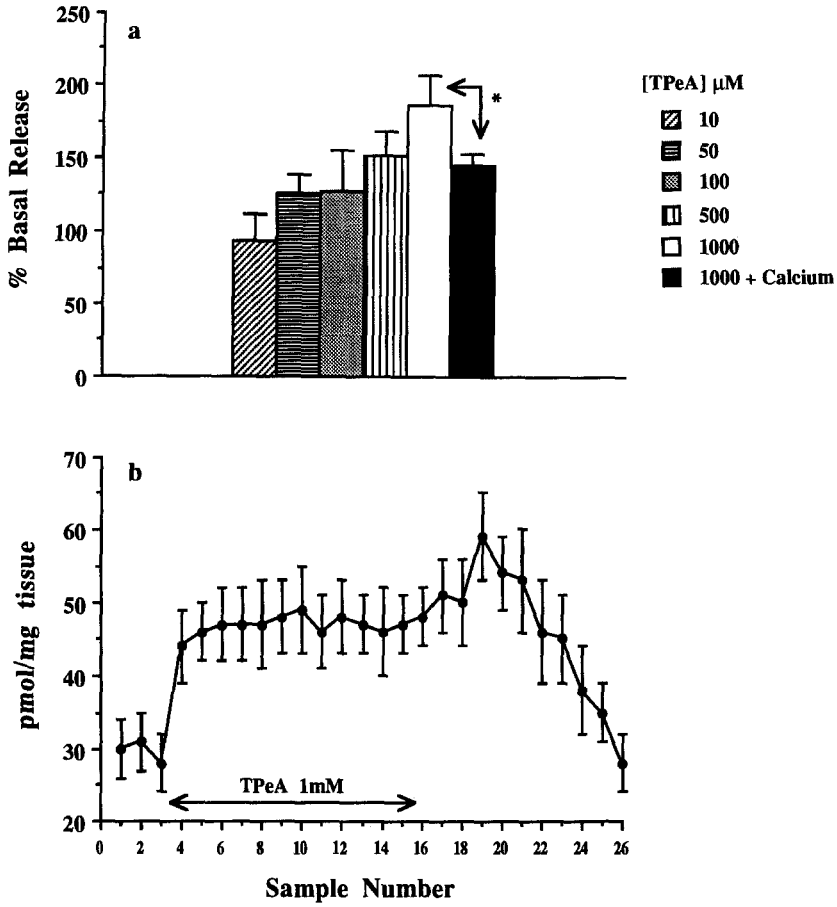

Fig. 1. a Effect of TPeA (2 minute pulses) on glutamate release in NACSF (filled bar) and Ca^{2+}-free ACSF (hatched bars). Mean ± sem. $*p < 0.05$ $n = 5–7$. **b** Effect of a 30 minute pulse of TPeA (1mM) on glutamate release in Ca^{2+}-free ACSF. Mean ± sem, $n = 10$

(Barnes et al., 1989). In addition, glutamate release was evoked by TPeA in Ca^{2+}-free ACSF in a concentration-dependent manner, and at 1mM the release in Ca^{2+}-free ACSF was significantly greater than in NASCF ($P < 0.05$, Fig. 1a). Repeated stimulation (× 3) with TPeA in NASCF exhibited no decay in glutamate release (100, 105 and 104% with respect to the first pulse, Fig. 4a). Indeed, in Ca^{2+}-free ACSF glutamate release could be evoked by 1mM TPeA for at least 30 minutes without diminution in the level of release (Fig. 1b).

Effect of K^+ channel opening agents on K^+- and TPeA-evoked release of glutamate

Cromakalim (0.5–4mM) reduced glutamate release evoked by 35 and 60mM K^+ in a concentration-dependent manner (Figs. 2a and 2b). Pinacidil (1 and 2mM) also inhibited K^+-evoked release, but to a lesser extent (Figs. 3a and 3b). Whereas the effect of pinacidil perfused during the second pulse appeared to continue to inhibit release in the third pulse, suggesting a slow washout of the drug, there was a slight enhancement of glutamate release in the third pulse following cromakalim (1mM) with 60mM K^+ ($P < 0.05$, Fig. 2b). Neither cro-

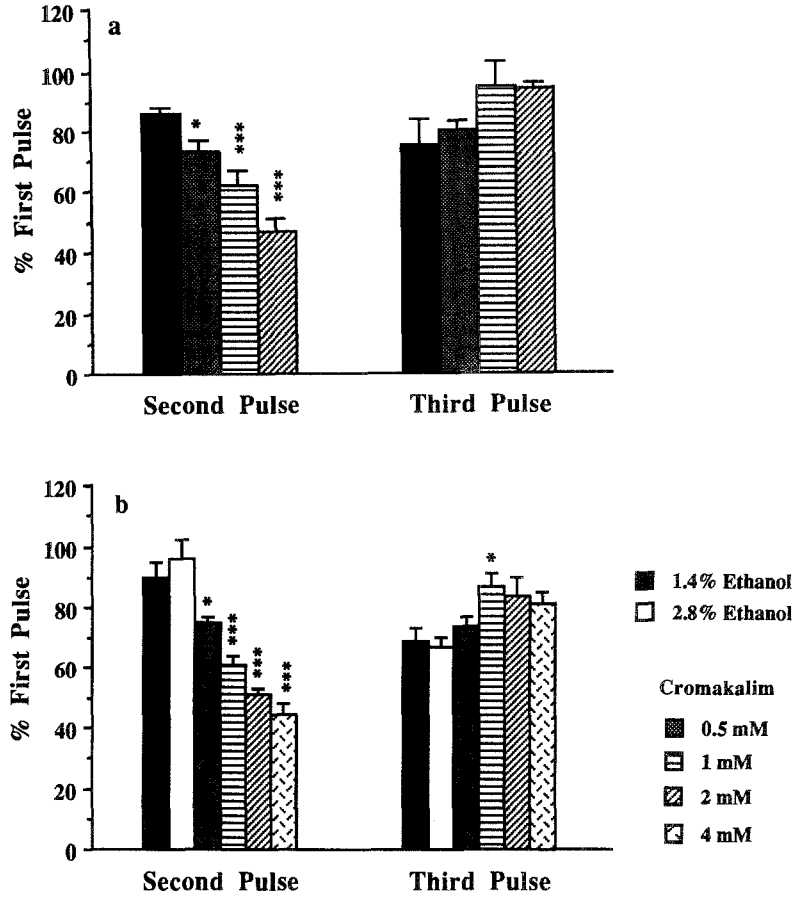

Fig. 2. Effect of cromakalim on (**a**) 35mM and (**b**) 60mM K^+-evoked release of glutamate with respect to control experiments containing vehicle (ethanol 1.4 and 2.8%). Mean ± sem, $n = 6$, $*p < 0.05$, $***p < 0.001$

makalim nor pinacidil altered basal glutamate levels, and both ethanol and DMSO were without effect on basal or K^+-evoked release (data not shown). However, ethanol (1.4%) significantly enhanced the TPeA-evoked release of glutamate in both NACSF ($P < 0.001$) and Ca^{2+}-free ACSF ($P < 0.01$, Figs. 4a and 4b).

Cromakalim (1mM) significantly reduced TPeA-evoked (1mM) glutamate release in Ca^{2+}-free and Ca^{2+}-replete conditions ($P < 0.001$ in both cases). This inhibition of release persisted in the third pulse (Figs. 4a and 4b). Pinacidil (2mM) also inhibited TPeA-evoked release in Ca^{2+}-replete ($P < 0.01$) but not in Ca^{2+}-free ACSF (Figs. 5a and 5b).

Discussion

Release of endogenous glutamate from cerebellar slices can be stimulated by K^+ channel blockade in addition to elevated K^+ concentrations. It is recognised, however, that the mechanisms of action are different. Tibbs et al., (1989) suggested that in synaptosomal preparations K^+ channel blockade causes repetitive

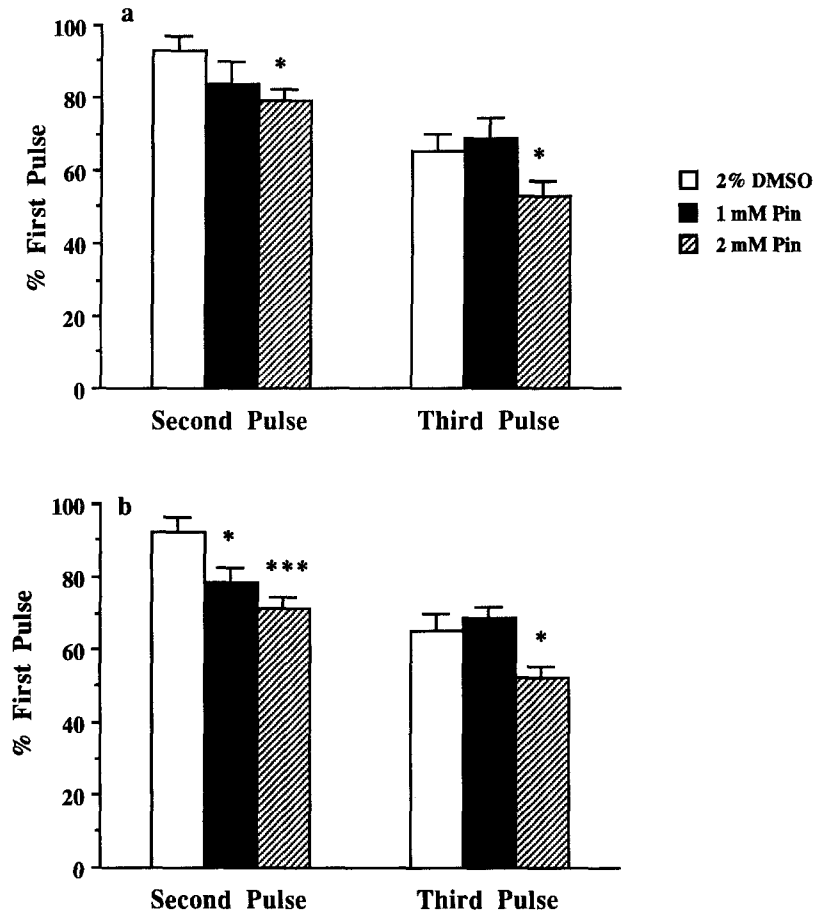

Fig. 3. Effect of pinacidil on (**a**) 35mM and (**b**) 60mM K^+-evoked release of glutamate with respect to control experiments containing vehicle (2% DMSO) Mean ± sem, $n = 6$, $*p < 0.05$, $***p < 0.001$

opening of Na^+ voltage-operated channels (VOCs) which induces a transient opening of Ca^{2+} VOCs, whereas elevated K^+ concentrations induce a synchronous activation of Ca^{2+} channels which is not that dependent on Na^+ channel activity. It appears that the K^+-evoked release of glutamate is not inhibited by TTX (Tibbs et al., 1989; Dickie and Davies 1992) whereas TPeA- or 4-aminopyridine-evoked release is TTX sensitive (Barnes et al., 1989; Tibbs et al., 1989). The ability of TPeA to facilitate glutamate release from cerebellar slices in Ca^{2+}-free medium has been reported previously (Barnes et al., 1989). Published evidence suggests that this observed effect may be due to an inhibition or reversal of the Na^+-coupled plasma-membrane glutamate uptake carrier, brought about by an accumulation of intraneuronal Na^+ (Nicholls, 1989).

In cultured hippocampal neurones, Politi et al., (1989) reported an inhibition of cromakalim-induced current by tetraethylammonium (TEA). However, Alzheimer et al., (1989) observed no antagonist effects of TEA, 4-AP or tolbutamide on cromakalim-induced changes in the membrane potential of hippocampal slices, although cromakalim has been shown to inhibit K^+-evoked neuronal discharges in hippocampal neurones (Alzheimer and ten Bruggencate,

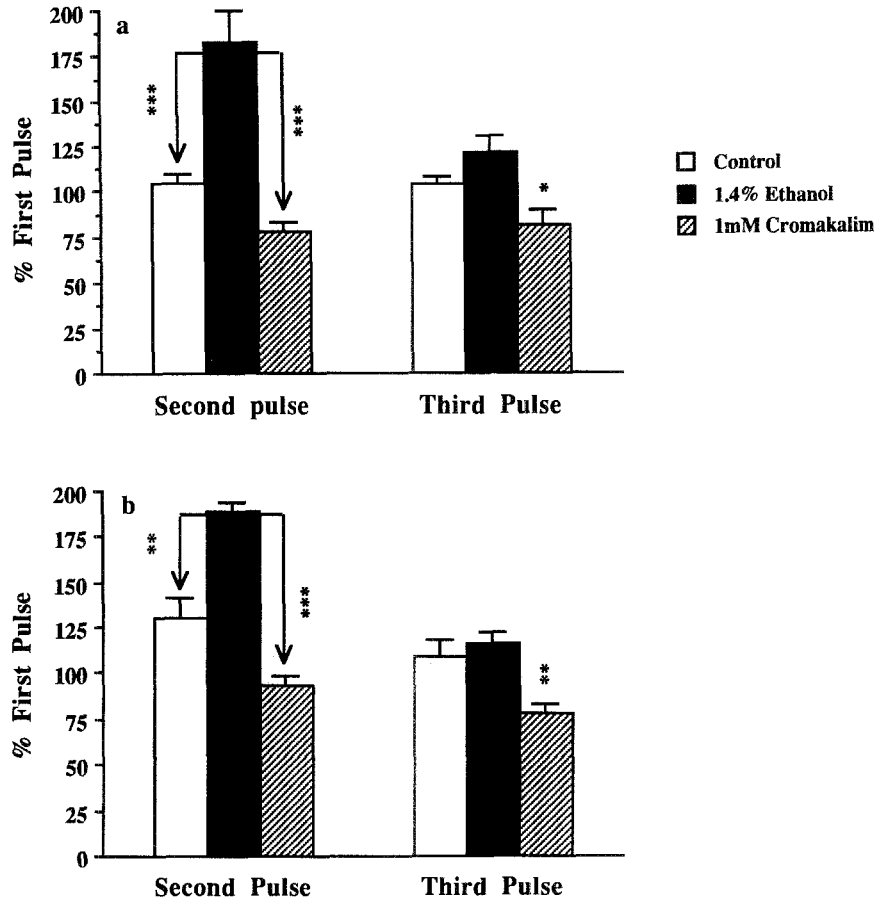

Fig. 4. Effect of ethanol (1.4%) and cromakalim (1mM) on TPeA-evoked (1mM) glutamate release in (**a**) normal and (**b**) Ca^{2+}-free ACSF. Mean ± sem, $n = 6$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

1988). The present results demonstrate that cromakalim can reduce TPeA-induced glutamate release in both Ca^{2+}-free and Ca^{2+}-replete conditions; pinacidil, however, was only effective in the presence of extracellular Ca^{2+}. This may reflect a facilitatory action on a Ca^{2+}-activated K^+ (K_{Ca}) conductance by cromakalim, as has been suggested for hippocampal neurones (Alzheimer et al., 1989), Recently the K_{Ca} channel blocker apamin has been shown to facilitate depolarisation-evoked release of glutamate in cerebellar slices (Dickie and Davies, 1993).

The results obtained regarding the inhibitory action of cromakalim and pinacidil on K^+-evoked glutamate release are in agreement with the inhibition of K^+-mediated effects by sulphonylurea K^+ channel activators in smooth muscle preparations (Hamilton et al., 1986; Weir and Weston, 1986 a,b; Allen et al., 1986). The reduction of the response to the third K^+ pulse following removal of pinacidil was probably, as with TPeA-evoked release, due to slow washout of the drug. With cromakalim, however, a slight potentiation of the third K^+ pulse was observed. One explanation is that cromakalim interferes with cytosolic Ca^{2+} homeostatic mechanisms, such as uptake into organelles (Cook and Quast, 1990)

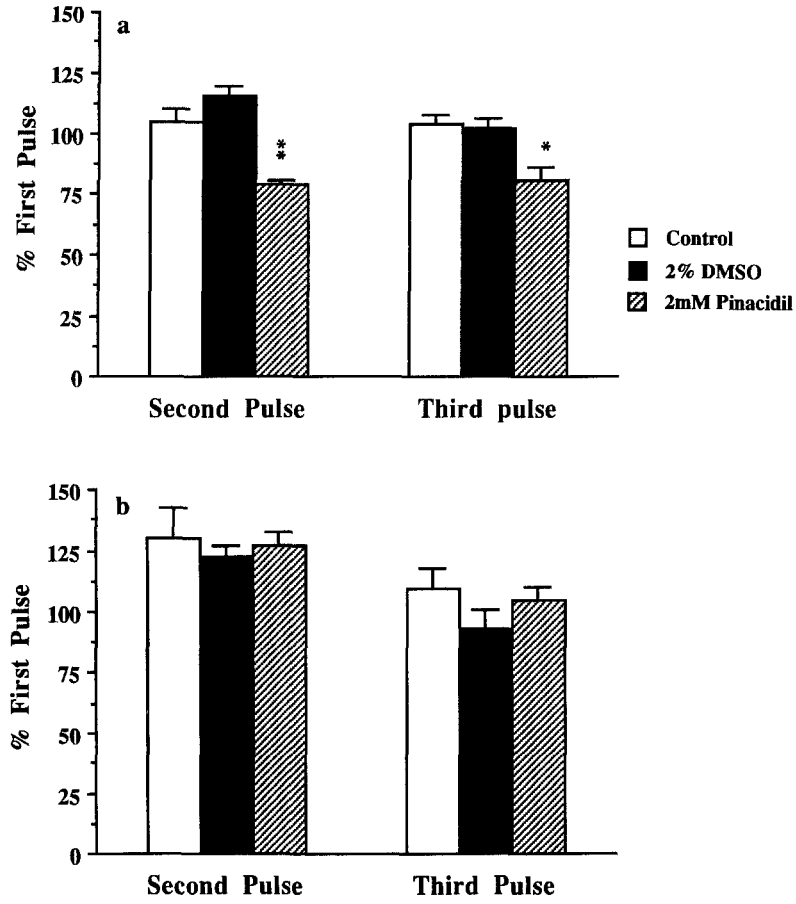

Fig. 5. Effect of DMSO (2%) and pinacidil (2mM) on TPeA-evoked (1mM) release of glutamate in (**a**) normal and (**b**) Ca^{2+}-free ACSF. Mean ± sem, $n = 6$, $*p < 0.05$, $**p < 0.01$ when compared to DMSO

leading to increased Ca^{2+}-dependent transmitter release. Whatever the mechanism, it would appear that this potentiation was antagonised by the enduring inhibitory (i.e K^+ channel opening) effects of the drug, as higher concentrations of cromakalim in the middle pulse reduced the potentiation of the third pulse.

Data regarding the effect of K^+ channel activators on transmitter release are rather sparse. In the periphery pinacidil has been shown to be effective in reducing [^{3}H]-noradrenaline release in guinea pig vasculature (Nedergaard, 1989). Hall and Maclagan (1988) suggested an effect on acetylcholine (ACh) release in the guinea pig trachea due to differences in the inhibition of nerve stimulated responses when compared with responses to exogenous ACh. McCaig and DeJonckheere (1989) concluded that cromakalim does not act at the cholinergic neuroeffector junction, but does not have an inhibitory effect on either the pre- or postganglionic nerve fibres.

The substantia nigra has been identified as containing the highest concentration of glibenclamide-sensitive binding sites in the CNS (Mourre et al., 1989). The release of labelled GABA from nigral slices can be enhanced by K^+_{ATP} channel blocking agents and inhibited by K^+_{ATP} channel activators (Amoroso

et al., 1990). The same group demonstrated the concomitant dose-dependent enhancement of ^{86}Rb efflux by K^+_{ATP} activators (Schmid-Antomarchi et al., 1990). Both the changes in transmitter release and ^{86}Rb efflux required remarkably low concentrations ($< 10^{-6}M$) of drug. This contrasts with the findings of Nelson (1989) who failed to observe changes in ^{86}Rb efflux in slice or synaptosomal preparations obtained from rat cortex and hippocampus, using higher concentrations ($10^{-4}M$). Although the results in this present paper regarding inhibition of neurotransmitter release concur with those of Schmid-Antomarchi and colleagues (1990) it appears that far higher concentrations are required.

The anoxia-induced depolarisation of hippocampal CA3 neurones has been shown to occur as a consequence of enhanced release of glutamate (Ben-Ari, 1990a). This depolarisation can be potentiated by the K^+_{ATP} channel blocker glibenclamide and attenuated by the K^+_{ATP} channel activators galanin, diazoxide and somatostatin (Ben-Ari, 1990a; Ben-Ari et al., 1990). A high density of glibenclamide binding sites on hippocampal mossy fibre terminals (Mourre et al., 1989) suggests a role for K^+_{ATP} channels in modulating glutamate release under anoxic conditions (Ben-Ari, 1990a; Ben Ari et al., 1990). The inhibition of depolarisation-evoked release of glutamate from cerebellar slices by cromakalim and pinacidil suggests that the K^+_{ATP} channels in the molecular layer of the cerebellum may be associated with glutamatergic parallel fibre nerve terminals of the granule cells. This concurs with binding studies in the granule cell deficient weaver mutant mouse strain, which has a greatly reduced K^+_{ATP} channel population in the cerebellar molecular layer (Mourre et al., 1990). However, it is unlikely that the channels are intimately associated with release of transmitter glutamate as in the hippocampus since higher concentrations are required.

One consequence of using high concentrations of K^+ channel activators is the correspondingly high concentrations of vehicle required. In control experiments 2% DMSO did not affect glutamate release. However, ethanol (1.4%) significantly increased TPeA-induced release but not K^+-evoked release. Ethanol has been reported to reduce depolarisation-induced uptake of Ca^{2+} into synaptosomes (Daniell et al., 1987) and inhibition of Ca^{2+} influx has been shown to enhance the TPeA-evoked release of glutamate (Barnes et al., 1989). However, as ethanol enhances TPeA-evoked release in the absence of extracellular Ca^{2+} it is possible that inhibition of Na^+ exchange processes by ethanol (Michaelis et al., 1983) potentiates the TPeA-induced enhancement of intracellular Na^+ and thus potentiates Ca^{2+}-independent release. In support of this we have observed that TPeA-evoked release of glutamate from cerebellar slices is TTX sensitive and therefore dependent on an enhancement of intracellular Na^+ (Barnes et al., 1989) whereas K^+-evoked release is not affected (Dickie and Davies, 1992).

The inhibition of depolarisation-evoked release of glutamate from cerebellar slices by cromakalim and pinacidil raises the likelihood that the K^+_{ATP} channels in the molecular layer of the cerebellum may be associated with glutamatergic parallel fibre nerve terminals; however, glutamate release from cerebellar slices would appear to be much less sensitive to K^+_{ATP} channel activators than hippocampal slices (Ben-Ari, 1990a), a brain region particularly sensitive to anoxia (Krnjevic, 1990). It has been proposed that activation of hippocampal K^+_{ATP} channels may provide a novel neuroprotective strategy during hypoxic

episodes (Ben-Ari, 1990b). Thus, dose-dependent specificity within distinct populations of central glutamatergic neurons may have potential therapeutic benefit.

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