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# Topiramate hyperpolarizes and modulates the slow poststimulus AHP of rat olfactory cortical neurones *in vitro*

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**1** The effects of the novel antiepileptic drug topiramate (TPM) were investigated in rat olfactory cortex neurones *in vitro* using a current/voltage clamp technique.

**2** In 80% of recorded cells, bath application of TPM (20 $\mu$M) reversibly hyperpolarized and inhibited neuronal repetitive firing by inducing a slow outward membrane current, accompanied by a conductance increase. The response was reproducible after washout, and was most likely carried largely by $K^+$ ions, although other ionic conductances may also have contributed.

**3** In 90% of cells, TPM (20 $\mu$M) also *enhanced* and prolonged the slow ($Ca^{2+}$-dependent) poststimulus afterhyperpolarization (sAHP) and underlying slow outward tail current ($sI_{AHP}$). This effect was due to a selective enhancement/prolongation of an underlying L-type $Ca^{2+}$ current that was blocked by nifedipine (20 $\mu$M); the TPM response was unlikely to involve an interaction at PKA-dependent phosphorylation sites.

**4** The carbonic anhydrase (CA) inhibitor acetazolamide (ACTZ, 20 $\mu$M) and the poorly membrane permeant inhibitor benzolamide (BZ, 50 $\mu$M) both mimicked the membrane effects of TPM, in generating a slow hyperpolarization (slow outward current under voltage clamp) and sAHP enhancement. ACTZ and BZ occluded the effects of TPM in generating the outward current response, but were additive in producing the sAHP modulatory effect, suggesting different underlying response mechanisms.

**5** In bicarbonate/$CO_2$-free, HEPES-buffered medium, all the membrane effects of TPM and ACTZ were reproducible, therefore not dependent on CA inhibition.

**6** We propose that both novel effects of TPM and ACTZ exerted on cortical neurones may contribute towards their clinical effectiveness as anticonvulsants.
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**Keywords:** Topiramate; acetazolamide; benzolamide; olfactory cortical brain slices; slow afterhyperpolarization current ($sI_{AHP}$); intracellular recording; voltage clamp

**Abbreviations:** ACTZ, acetazolamide; sAHP, slow afterhyperpolarization; cAMP, cyclic adenosine monophosphate; BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′, *N*′-tetraacetic acid; BZ, benzolamide; DMSO, dimethyl sulphoxide; EGTA, ethylene glycol-bis(b-aminoethylether)-*N*,*N*,*N*′, *N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-propanesulphonic acid; HVA, high voltage-activated; PKA, protein kinase A; TPM, topiramate; TTX, tetrodotoxin

## Introduction

In the past decade, a number of 'newer generation' anti-epileptic drugs (AEDs) have been introduced therapeutically, including lamotrigine, gabapentin, felbamate, oxcarbazepine, tiagabine, levetiracetam, topiramate (TPM) and zonisamide, which have proved highly effective against various clinical seizure types and offer more favourable pharmacokinetics, and less troublesome side effects or adverse drug interactions than with other AEDs (Asconape, 2002). Following clinical trials, these drugs have become available for drug therapy of epilepsy, either singly or in combination with established AEDs; the precise mechanism of anticonvulsive action of some of these novel AEDs, however, is still uncertain (for a review, see Kwan *et al*., 2001).

TPM (2,3 : 4,5-bis-*O*-(1-methylethylidene)-$\beta$-D-fructopyranose sulphamate; Topamax®) is currently used as an additive treatment for adult patients with refractory partial and secondarily generalized seizures (Bauer & Schwalen, 2000); it has also been found useful as adjunctive therapy in children, adolescents and young adults with partial-onset seizures or Lennox–Gastaut syndrome (Coppola *et al*., 2001; 2002). A recent monotherapy study indicates that low-dose (100 mg $day^{-1}$) TPM is effective in treating newly diagnosed epilepsy (Privitera *et al*., 2003). It is a structurally-unique drug containing an essential *O*-sulphamate moiety (Maryanoff *et al*., 1987; 1998) that was originally synthesized as a possible blocker of gluconeogenesis. In traditional animal seizure models, TPM suppressed maximal electroshock seizures, but only weakly inhibited pentylenetetrazol (PTZ)-induced clonic seizures, being similar in effectiveness to phenytoin and

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carbamazepine (Shank *et al*., 1994). More recent clinical studies (Herranz, 2000) suggested that TPM may be the most potent of the newer generation of antiepileptic drugs in treating refractory epilepsies.

Thus far, multiple mechanisms underlying the anticonvulsant action of TPM have been proposed, based on experiments carried out on a variety of *in vitro* neuronal preparations (e.g. Kawasaki *et al*., 1998; DeLorenzo *et al*., 2000); these main mechanisms include: use-dependent inhibition of voltage-dependent $Na^+$ channels (Wu *et al*., 1998; Taverna *et al*., 1999) and L-type $Ca^{2+}$ channels (Zhang *et al*., 2000), potentiation of $\gamma$-aminobutyric acid type A ($GABA_A$)-induced $Cl^-$ currents (White *et al*., 2000), and reduction of glutamate-mediated excitation by antagonizing $\alpha$-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kainate (but not *N*-methyl-D-aspartate (NMDA)) receptors (Gibbs *et al*., 2000; for a recent review, see Angehagen *et al*., 2003). However, in some *in vitro* studies, TPM had no apparent effect on intrinsic membrane properties, evoked $Na^+$ or $Ca^{2+}$ spikes, induced repetitive firing or synaptic transmission (Hanaya *et al*., 1998; Jahromi *et al*., 2000), which would contrast with the generally accepted mechanisms of action proposed above. Interestingly, since TPM crosses cell membranes readily, it has been suggested that its complex modulatory (and often variable) effects on certain receptor/ion channels may be mediated by selective binding of the drug to *intracellular* phosphorylation sites on the receptor/ion channel complexes in the *de*phosphorylated state (Shank *et al*., 2000). The effective bath concentrations of TPM in these experiments ranged from around 10 to 100 $\mu$M, which is in accordance with free serum levels of TPM observed in patients receiving adjunctive treatment for seizure disorders ($2.5–35\,mg\,l^{-1} \equiv 7.4–103\,\mu M$; TPM molecular wt. = 339; Wolf *et al*., 2000). By contrast, TPM was shown to be inactive on a variety of other receptor-binding, neurotransmitter uptake and ion channel sites, although it did exert some carbonic anhydrase (CA) inhibitory activity (Maryanoff *et al*., 1987; Shank *et al*., 2000; Casini *et al*., 2003); the relevance of this property to the general anticonvulsant action of TPM, however, remains uncertain (cf. Herrero *et al*., 2002).

Apart from its antiepileptic properties, TPM is also effective in treating other neurologic and neuropsychiatric disorders (e.g. bipolar disorder, Suppes (2002); neuralgia, Zvartau-Hind *et al*. (2000); migraine, Pascual (1999); bulimia, McElroy *et al*. (2003) and post-traumatic stress disorder, Berlant & van Kammen (2002)); this surprisingly wide spectrum of activity suggests that other ion channel, neurotransmitter or biochemical, mechanisms may also be targeted by this novel drug. A better understanding of the mode(s) of action of TPM would therefore seem warranted, not only in an attempt to explain its multiple activity, but also in the hope of identifying possible new target sites for future antiepileptic drug development. In the present work, we have used *in vitro* slices of rat olfactory cortex (a convenient brain slice model; Constanti *et al*., 1993) to study the direct effects of TPM on neuronal membrane properties and post-stimulus afterpotentials recorded under current-clamp or voltage-clamp conditions, and to examine whether these effects were indeed related to CA inhibition; the olfactory cortical brain area was of particular interest, in view of its known susceptibility to limbic epileptogenesis (Löscher & Ebert, 1996).

We describe here two main effects of TPM on these cortical cells: induction of a slow membrane hyperpolarization and enhancement and prolongation of a slow afterhyperpolarization (sAHP) that follows a burst of action potentials. We suggest that these effects could make a major contribution towards the anticonvulsant effectiveness of this drug in the brain. Preliminary accounts of our data have been presented to the British Pharmacological Society (Russo & Constanti, 2002; Russo *et al*., 2003).

## Methods

### *Preparation and storage of brain slices*

Experiments were carried out using transverse, rostro-caudal slices of olfactory (piriform) cortex ($\sim$450 $\mu$m thick; 6–10 slices per animal) prepared from adult Wistar rats (150–200 g; either sex). Animals were decapitated after deep halothane anaesthesia in accordance with the Home Office Animals (Scientific Procedures) Act (1986), and the brain rapidly removed, hemisected and placed into ice-cold (4°C) oxygenated Krebs' solution. Serial slices were then sectioned using a Campden Vibroslice/M tissue cutter (Campden Instruments, U.K.), as previously described (Constanti *et al*., 1993), and stored in oxygenated Krebs solution at 32°C for at least 30 min, before transferring to a Perspex recording chamber, where they were superfused continuously (held completely submerged) at $\sim 10\,ml\,min^{-1}$ with prewarmed oxygenated Krebs solution at 29–30°C. The composition of the normal Krebs solution was (in mM): 118 NaCl, 3 KCl, 1.5 $CaCl_2$, 25 $NaHCO_3$, 1 $MgCl_2 \cdot 6H_2O$ and 11 D-glucose (bubbled with 95% $O_2$–5% $CO_2$, pH 7.4). Bicarbonate-free HEPES-buffered Kreb's solution contained (mM): 133 NaCl, 3 KCl, 1.5 $CaCl_2$, 1 $MgCl_2 \cdot 6H_2O$, 10 HEPES and 11 D-glucose (bubbled with 100% $O_2$, pH 7.4 adjusted with 1 N NaOH).

### *Recording*

Intracellular current-clamp or voltage-clamp recordings were made from the periamygdaloid area of the slices within the piriform cortical cell layers II–III, using glass microelectrodes filled with 4 M potassium acetate (tip resistances 40–70 MΩ) coupled to an Axoclamp 2A sample-and-hold preamplifier (2–3 kHz switching frequency, 30% duty cycle; Axon Instruments, CA, U.S.A.). In order to minimize the outward $K^+$ conductances, some experiments under voltage clamp were carried out using electrodes filled with 3 M caesium acetate (tip resistances 30–50 MΩ), and in the presence of 1 $\mu$M tetrodotoxin (TTX), to block $Na^+$ spike generation. Membrane input resistance and firing behaviour were assessed by injecting positive or negative current pulses of varying intensities ($\sim$0.25–3 nA; 160 ms), and the resulting electrotonic potentials or evoked action potentials recorded, respectively. First spike amplitudes were always measured (from baseline level) under 'bridge' recording mode to avoid sampling limitations of the discontinuous sample-and-hold preamplifier. In current-clamp experiments, the membrane potential was usually maintained at $-70$ mV (near firing threshold) by applying a steady depolarizing current, while in voltage-clamp recordings, the holding membrane potential was routinely set at $-70$ mV. The steady voltage level recorded at the soma following voltage

step commands (in TTX) was always used to construct current–voltage (*I–V*) curves. Acceptable recordings typically remained stable for periods of 1–5 h. Sampled membrane current and voltage signals were monitored on a storage oscilloscope and a Gould 2400 ink-jet chart recorder (rise time $<8$ ms for a square wave input), and also fed directly to a computer (Viglen Ltd, U.K.) *via* a Digidata 1200 analogue-to-digital interface (Axon Instruments, CA, U.S.A.), using pCLAMP 6.03 software (Axon Instruments) for hard disk recording and off-line analysis. Voltage-clamp currents following step commands were not corrected for leakage or capacitative currents. All measurements were made before, during and after bath superfusion (bath-exchange time $\sim 30$ s) of pharmacological agents, so that each neurone served as its own control. Unless otherwise stated, each experimental sequence described below was repeated at least three times on different cells. Data are expressed as means $\pm$ s.e.m., and, when appropriate, statistical significance of the differences between data group means was evaluated using a standard Student's *t*-test.

### *Drugs*

TPM (Johnson & Johnson Pharmaceutical Research & Development, L.L.C., Raritan, NJ, U.S.A.) was prepared as a 10 mM stock solution in distilled water, and was stored for up to 7 days at 4°C without apparent loss of activity. Acetazolamide (ACTZ), $\beta$-parachlorophenyl-$\gamma$-aminobutyric acid [(−)baclofen], (−)-bicuculline methiodide, forskolin, nifedipine, ouabain and TTX were all purchased from Sigma-Aldrich Co. Ltd, Poole, U.K., and routinely prepared as stock solutions in distilled water and stored at 4°C; drugs were subsequently diluted in Krebs solution immediately before use. Benzolamide was a gift from Professor E.R. Swenson, School of Medicine, University of Washington, Seattle, WA, U.S.A. All other reagents were obtained from BDH (Analar grade). Nifedipine and benzolamide were dissolved in DMSO and ethanol, respectively, and then diluted in Krebs just before use; the final bath concentrations of DMSO (up to 0.5%) or ethanol (0.1%) had no deleterious effects on membrane properties. Due to their light sensitivity, nifedipine, ACTZ and ouabain were weighed and dissolved in semi-darkness, and then stored in containers wrapped in silver foil to exclude light. Exposure to ambient light during application of these drugs to cells was also minimized. The results presented below are based on recordings obtained from $\sim 150$ cortical neurones in $\sim 100$ piriform cortex slices.

## Results

### *Characterization of slow hyperpolarizing action of TPM*

TPM was tested on 37 olfactory cortical neurones recorded under current clamp; their average resting potential and input resistance (calculated from $\leqslant 20$ mV hyperpolarizing electrotonic potentials) were $-82.2 \pm 0.3$ mV and $41.1 \pm 1.6$ MΩ, respectively. In 31 of 37 (84%) cells maintained at $-70$ mV membrane potential by positive current injection, bath application of TPM (20 $\mu$M, 20–50 min) induced a slow hyperpolarization (mean peak amplitude $= 9 \pm 2$ mV, significantly different from the rest level; $P<0.001$), which reached a plateau between 8 and 15 min after drug onset, and was accompanied by a significant decrease in input resistance (mean $= 26 \pm 7\%$; $P<0.001$); this was indicated by the reduction in amplitude of the hyperpolarizing electrotonic potentials evoked by negative current injection (Figure 1a, b). On washout of TPM, both the membrane potential and input resistance slowly recovered to control levels over 15–30 min; the hyperpolarizing response was quite reproducible, provided adequate washout time was allowed. A similar slow hyperpolarization induced by 100 $\mu$M TPM was recently reported by Herrero *et al*. (2002) in hippocampal CA1 neurones, and attributed to an increase in $K^+$ conductance (see below). Hyperpolarizing responses to TPM were dose-dependent within the range of 10–50 $\mu$M TPM; in general, the higher (50 $\mu$M) dose of TPM produced membrane actions that were more prominent and longer lasting, so that full recovery of the drug-induced changes were not always observed despite 1 h of washout. A standard 20 $\mu$M dose of TPM was therefore used in all subsequent experiments in order to facilitate reproducibility and multiple TPM comparisons in any given cell.

On testing cell firing properties by injecting a brief (160 ms, $+0.75$ nA) depolarizing current stimulus (after correcting for change in membrane potential produced by TPM, by positive current injection), TPM induced a significant reduction in the number of action potentials elicited during the pulse (mean number of control spikes $= 7.2 \pm 1.7$, decreased to $2.2 \pm 0.9$ spikes in TPM; $P<0.05$, $n=22$; $\sim 70\%$ reduction), indicating a decreased cell excitability, along with a small ($\sim 18\%$) reduction in peak amplitude of the first spike overshoot in the train (mean $= 19.8 \pm 3.5$ mV control vs $16.2 \pm 2.5$ mV in TPM; $P<0.05$, $n=22$) (cf. Wu *et al*., 1998); the activation threshold for triggering action potentials (typically between $\sim -60$ and $-65$ mV), however, was not notably altered. These effects were fully reversed after 40 min washout (Figure 1c). Interestingly, in six neurones tested under current clamp, the membrane potential, input resistance and number of evoked spikes were not notably affected by TPM, suggesting that the action of the drug may vary depending on neurone type (i.e. interneurone or deep pyramidal cell; Libri *et al*., 1994) or, possibly, cell metabolic status (see below).

Under a voltage clamp at $-70$ mV holding potential, the slow membrane hyperpolarization induced by TPM manifested as a slow outward shift in baseline current, peaking over a 10 min application, and slowly reversing on washout (30 min) (Figure 2a); the TPM current was insensitive to 1 $\mu$M TTX ($n=17$). The mean outward current recorded in 32 of 34 cells was $0.36 \pm 0.26$ nA (two cells tested in voltage clamp failed to respond to TPM – see above). On repeating the TPM application at different steady holding potentials (between $-70$ and $-110$ mV; $n=6$), the slow outward current decreased in amplitude with increasing hyperpolarization (as might be expected if it were principally mediated by $K^+$ ions; Herrero *et al*., 2002), but never showed a clear reversal to an inward current shift, even at $-110$ mV (Figure 2b). Accordingly, current–voltage (*I–V*) curves constructed (in TTX) using 1.5 s voltage jumps in the presence and absence of TPM failed to intersect at negative potentials (Figure 3a); this suggests that either a mixture of ionic conductances and/or some electrogenic pump mechanism might be involved in mediating the TPM effect in these cortical cells. As a control, the action of TPM was compared with that of the $GABA_B$ receptor agonist baclofen (20 $\mu$M), which is known to selectively activate a

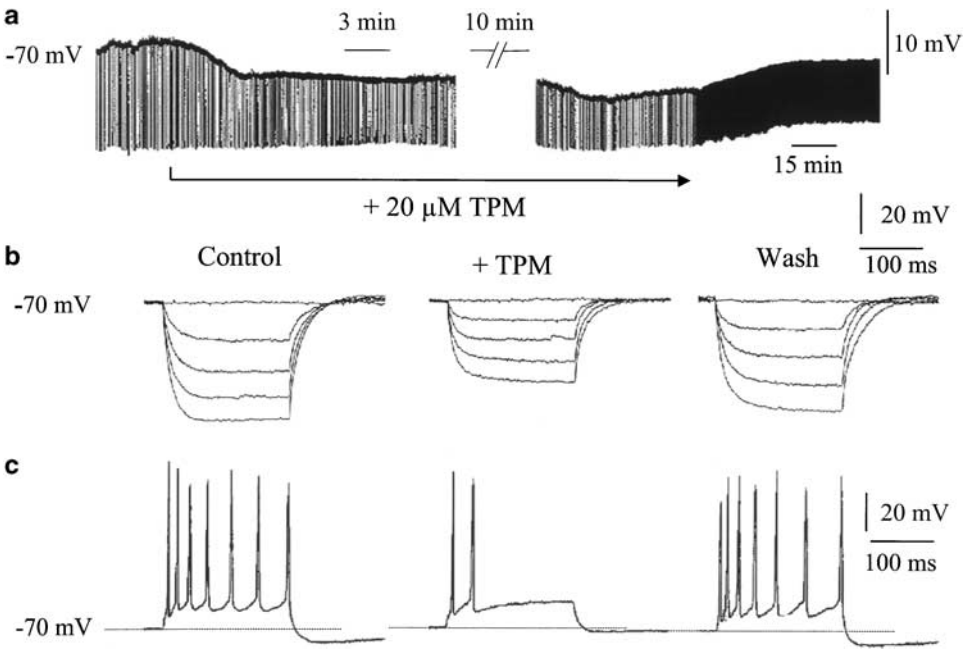


**Figure 1** Effects of TPM on the membrane properties of an olfactory cortical neurone. (a) Slow membrane hyperpolarization accompanied by a decrease in membrane input resistance induced by bath application of TPM (20 $\mu$M; 40 min) from a holding membrane potential of −70 mV (maintained by steady current injection). Negative current pulses (−0.5 nA, 160 ms) were injected every 2 s throughout. (b) Superimposed electrotonic potentials evoked by injecting brief (160 ms; −0.25 nA) hyperpolarizing current pulses. TPM induced a clear and reversible decrease in input resistance. (c) Electrotonic response to injection of a depolarizing current pulse (160 ms; + 0.75 nA). TPM decreased the number of action potentials elicited during the pulse, indicating a reduction in cell excitability (corrected for change in membrane potential produced by TPM, by positive current injection).

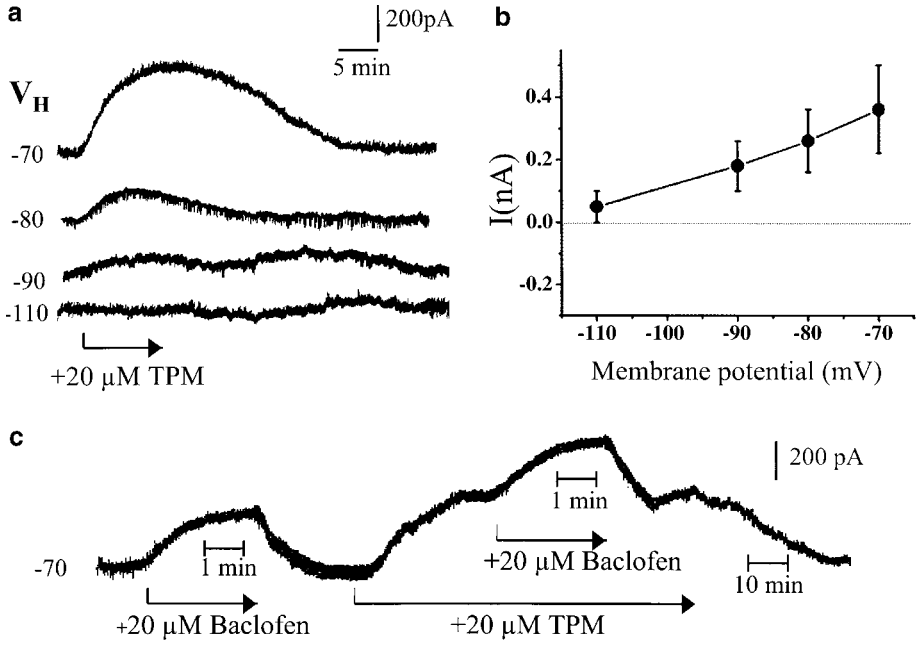


**Figure 2** (a) TPM-induced slow outward currents recorded in a single neurone voltage clamped at various holding potentials from −70 to −110 mV. In each case, TPM (20 $\mu$M) was applied for 10 min, followed by a 30 min washout period; note the progressive reduction in current amplitude at more negative potentials, but lack of reversal to a slow inward current, even at −110 mV. (b) Corresponding plot of slow current amplitude against holding potential (points represent means ± s.e.m., $n = 6$; currents were measured from peak to baseline level). (c) Different neurone clamped at −70 mV; baclofen (20 $\mu$M; 3 min) applied initially in control Krebs solution, produced a steady outward current with a relatively rapid rate of onset/offset. Addition of TPM (20 $\mu$M; 40 min) induced a similar amplitude outward current, but did not occlude the response to baclofen when applied in combination (note a faster chart speed during baclofen responses).

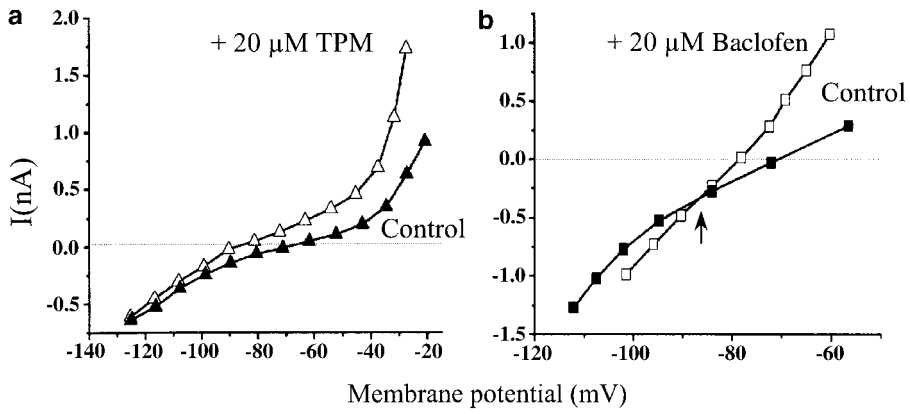


**Figure 3** Steady-state current–voltage (*I–V*) relationships obtained under voltage clamp (in TTX), showing the peak amplitude of membrane currents evoked by 1.5 s depolarizing or hyperpolarizing voltage steps from a holding potential of −70 mV (ordinates) vs membrane potential (abscissae). Filled symbols: currents recorded in control solution; open symbols: currents measured during drug application (10 min). (a) *I–V* plot in the presence and absence of 20 $\mu$M TPM shows a characteristic upward shift with increase in slope (increased conductance) compared with control, at potentials between −80 and −20 mV, but no crossover at negative potentials. (b) (Different cell) *I–V* plot in the presence and absence of 20 $\mu$M baclofen also shows increase in slope, but intersects the control curve at ~−85 mV (estimated $E_{rev}$ for the baclofen-induced current; arrow), as might be expected from principal activation of a $K^+$-selective (GIRK) conductance mechanism.

G-protein coupled inward-rectifying $K^+$ conductance (GIRK) in these neurones (Malcangio *et al*., 1995) ($n=4$ cells) (Figure 2c). Although baclofen produced outward currents of similar amplitude to those of TPM, the baclofen-induced outward current was much faster in onset/offset, and was *not* occluded when superimposed at the peak of the TPM response, suggesting that the currents were mediated by *independent* ionic mechanisms; moreover, the (*I–V*) curves in the presence and absence of baclofen showed a clear crossover at negative potentials (Figure 3a), allowing a mean reversal potential for the baclofen-induced current to be estimated as $-90\pm5$ mV ($n=4$), which is close to the calculated $E_K$ of −95 mV in these cells (taking internal $K^+$ concentration as 130 mM; Harvey *et al*., 1974).

In the report of Herrero *et al*. (2002), the membrane hyperpolarization induced by 100 $\mu$M TPM in hippocampal CA1 neurones was abolished in the presence of 1 mM $Ba^{2+}$, a recognized blocker of both outward- and inward-rectifying $K^+$ conductances (Eaton & Brodwick, 1980). In our experiments, however, this high concentration of $Ba^{2+}$ only partially blocked the slow outward current induced by TPM (mean = 47.4±11.3% reduction, $n=4$; $P<0.001$), indicating that a $Ba^{2+}$-resistant component was also being activated (Figure 4a); the nature of this other ionic conductance remains unclear. In further tests, we found that the TPM current was unaffected by 200 $\mu$M $Cd^{2+}$ ($n=9$), indicating that it was not dependent on external $Ca^{2+}$ entry (i.e. not a $Ca^{2+}$-activated $K^+$ conductance: $I_{K(Ca)}$), (Figure 4b), although the induced release of $Ca^{2+}$ from intracellular stores cannot be excluded. Moreover, the current was unaffected by 10 $\mu$M bicuculline methiodide (in the presence of TTX, to prevent spontaneous epileptiform firing activity; $n=8$) (Figure 4c), thus ruling out the indirect involvement of $GABA_A$ receptors, as suggested by Kawasaki *et al*. (1998) for rat subicular neurones. Finally, blocking the $Na^+$–$K^+$–ATPase with 10 $\mu$M ouabain (10 min; $n=4$) also failed to affect the TPM-induced current, indicating that a $Na^+$–$K^+$ electrogenic pump mechanism was not being activated (cf. Gustafsson & Wigström, 1983). Ouabain also had little or no effect on the sAHP evoked by a burst of action potentials (cf. Schwindt *et al*., 1988), or on the ability of TPM to enhance/prolong it (see below).

### *Enhancement and prolongation of sAHP by TPM*

In the second main series of experiments, we aimed to further characterize the novel enhancing effect of TPM on the sAHP. A representative example of this phenomenon is shown in Figure 5. In control solution under current clamp, injection of a long (1.5 s) depolarizing current stimulus produced a burst of action potentials followed by an sAHP, due to activation of a $Ca^{2+}$-activated $K^+$ conductance (Constanti & Sim, 1987) (Figure 5a). In TPM (20 $\mu$M, 20 min, $n=33$) (after correcting for any change in membrane potential), there was a significant increase in the amplitude (~20%) (control mean = 10.5±3.4 vs 12.5±3.8 mV in TPM; $P<0.001$) and particularly, the duration (~63%) (3.39±0.48 s control vs 5.52±1.29 s in TPM; $P<0.001$; paired *t*-tests) of the sAHP, which was slowly reversed on washout (~60 min). There was also a dramatic increase in spike accommodation during the stimulus pulse. The corresponding slow outward tail currents underlying the sAHP ($sI_{AHP}$; Sah & Faber, 2002) were revealed using a 'hybrid' voltage-clamp protocol (Constanti & Sim, 1987), in which a manual switch to voltage clamp at −70 mV was made immediately following a 1.5 s depolarizing stimulus (Figure 5b). In TPM, the $sI_{AHP}$ tail was found to be significantly enhanced in amplitude (~37%) (269±137 pA control vs 369±142 pA in TPM; $P<0.001$) and prolonged in duration (~84%) (3.30±1.04 vs 6.07±1.16 s in TPM; $P<0.001$) ($n=14$). In this series, two of 16 neurones tested (~12%) failed to show an obvious change in the $sI_{AHP}$ magnitude or time course in TPM, although, interestingly, these cells still generated a slow outward current shift under voltage clamp; this suggests that the underlying response mechanisms involved in the two TPM effects are independent of each other.

In an attempt to understand the mechanism underlying the $sI_{AHP}$ enhancement by TPM, we recorded under voltage clamp at −70 mV in the presence of 1 $\mu$M TTX (to prevent fast $Na^+$ spike generation), and applied a large positive voltage jump

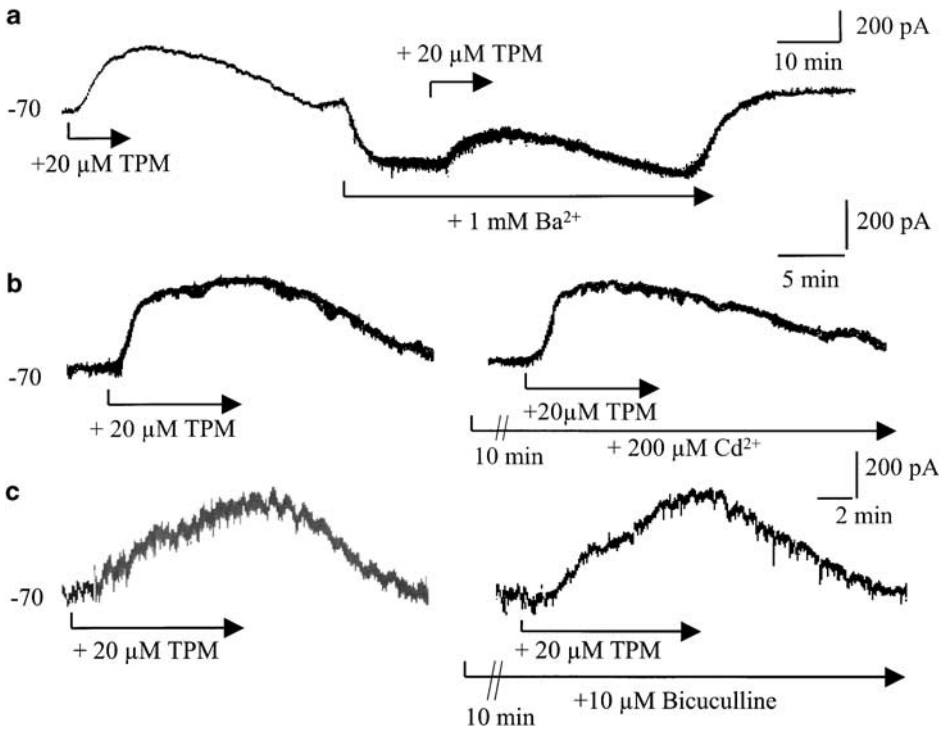


**Figure 4** Pharmacology of TPM-induced slow outward current recorded under voltage clamp (a–c, different neurones clamped at −70 mV). (a) Application of TPM (20 $\mu$M; 10 min) in the presence of 1 mM $Ba^{2+}$ (20 min) produced only a partial block of the TPM current (peak amplitude reduced by 48%). In contrast, 200 $\mu$M $Cd^{2+}$ (b) or 10 $\mu$M bicuculline (c) (10 min preapplications) failed to affect the TPM responses (note the different chart speeds in each experiment).

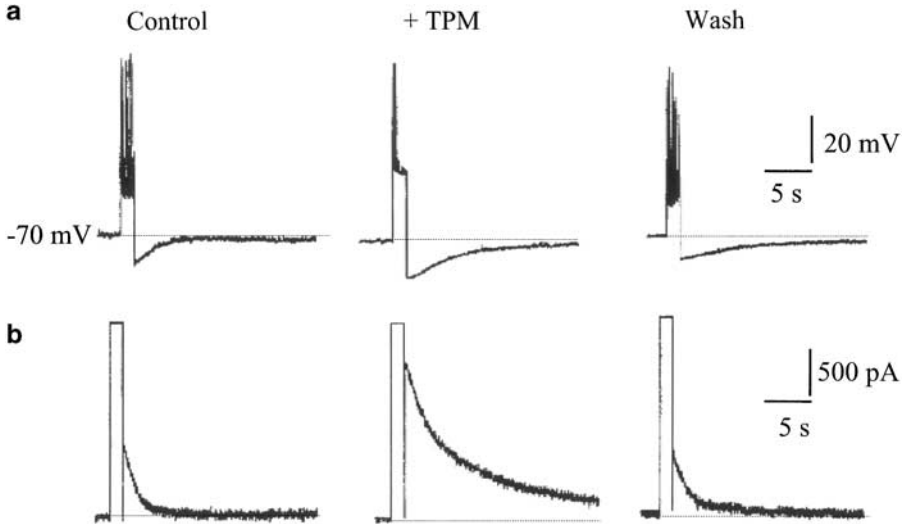


**Figure 5** TPM enhances and prolongs the poststimulus sAHP and s$I_{AHP}$ tail current. (a) Response to injection of a long (1.5 s; +2.0 nA) depolarizing current stimulus, which in control solution evokes a burst of action potentials, followed by a sAHP. In the presence of TPM (20 $\mu$M), there was an enhancement and dramatic prolongation of the sAHP, accompanied by a noticeable increase in spike accommodation during the pulse, that was reversible on washout (60 min) (membrane potential change in TPM corrected back to −70 mV, by steady positive current injection). (b) Corresponding outward tail current (s$I_{AHP}$) evoked using a 'hybrid' voltage-clamp protocol from a holding potential of −70 mV. Note that TPM enhanced and prolonged the time course of the s$I_{AHP}$. An outward baseline shift of 0.38 nA induced by TPM was omitted for clarity.

from −70 to −20 mV (1.5 s) to observe the outward current relaxations. Figure 6 shows such an experiment (superimposed records). In 20 K acetate-recorded cells, this protocol, in control solution, evoked a slow outward relaxation (1.77 ± 0.7 nA peak amplitude) and slow outward tail current (s$I_{AHP}$) (115 ± 23 pA peak, 3.37 ± 0.64 s duration), largely due to activation of the slow $Ca^{2+}$-activated $K^+$ conductance (Constanti & Sim, 1987). On applying TPM (20 $\mu$M; 20 min; $n = 17$), this outward relaxation was clearly increased in amplitude (2.23 ± 0.9 nA), along with an enhancement

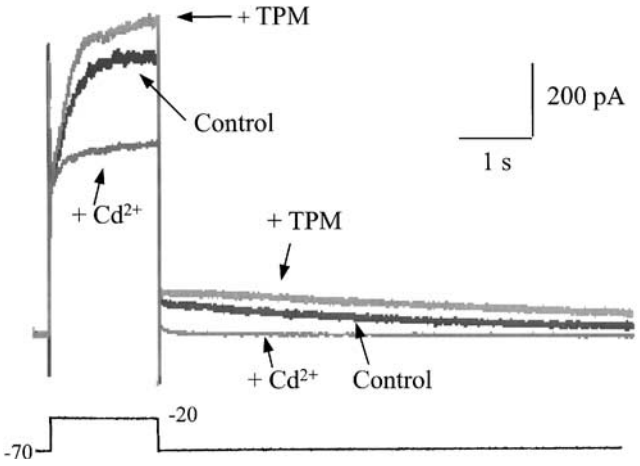


**Figure 6** (a) Effect of TPM on clamp currents evoked by a 1.5 s voltage command from a holding potential of −70 to −20 mV (in 1 $\mu$M TTX). Superimposed records show the outward current developed during positive command and the ensuing slow outward tail current ($sI_{AHP}$) in control Krebs solution then, after 10 min in 20 $\mu$M TPM. Note the potentiation of outward relaxation and after-tail current. Addition of $Cd^{2+}$ (200 $\mu$M) blocked both the extra outward current and $sI_{AHP}$ tail components induced by TPM. A steady outward current of 0.15 nA developed in TPM (omitted for clarity), along with a 27% increase in leak conductance measured around −70 mV.

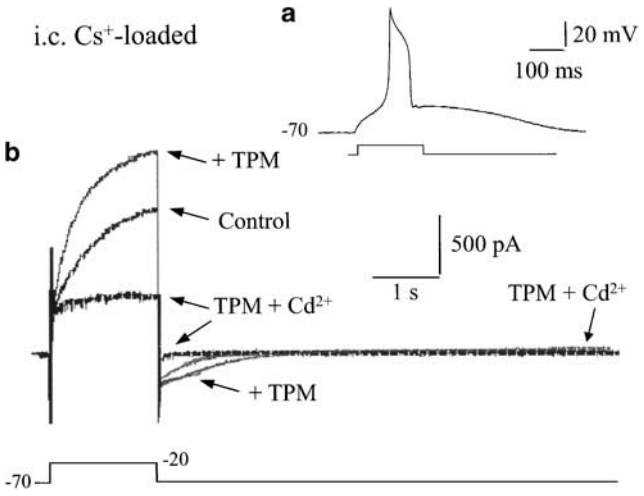


**Figure 7** Effects of TPM recorded in a $Cs^+$-loaded neurone (in 1 $\mu$M TTX). (a) Depolarizing electrotonic potential and action potential (evoked by a +0.3 nA, 160 ms current pulse) recorded 30 min after impalement of a cell with a 3 M Cs acetate-filled microelectrode; note the typically prolonged duration and slow rate of onset of the TTX-resistant ($Ca^{2+}$) spike, signifying partial blockade of outward (repolarizing) $K^+$ conductances. Note also the prominent slow depolarizing afterpotential (DAP) following the spike repolarization. The cell membrane potential was set at −70 mV by steady current injection. (b) (Same neurone under voltage clamp) Superimposed clamp currents recorded from −70 mV holding potential following a 1.5 s voltage step to −20 mV show the enhancement of residual outward relaxation in TPM (20 $\mu$M, 15 min), although an enhanced *inward* after-tail current was now revealed. Both outward relaxation and induced inward tail were abolished by $Cd^{2+}$ (200 $\mu$M, 10 min) (note, no steady outward current shift was produced by TPM in this $Cs^+$-loaded cell).

(153 ± 34 pA) and dramatic prolongation (7.83 ± 1.32 s) of the slow $sI_{AHP}$ after-tail current (means significantly different from control; $P<0.001$; paired *t*-tests); in the corresponding *I–V* relation (cf. Figure 3a), the action of TPM was manifested as a greater degree of outward current rectification (relative to control) over the potential range of −60 to −20 mV. On further addition of $Cd^{2+}$ (200 $\mu$M; $n=15$), both enhanced components were eliminated, confirming the $Ca^{2+}$ dependence of the extra TPM-induced current (Figure 6, lower record), and the strong outward rectification of the *I–V* plot was abolished (not shown). We noted that in 3/20 cells, the peak of the outward current relaxation in TPM was *smaller* than that observed in control, even though the $sI_{AHP}$ tail current was still enhanced; this could possibly indicate an interplay between inward and outward current components developing during the voltage jump, that were being differentially affected by TPM.

### *Intracellular $Cs^+$-loading experiments*

In the next set of experiments, we wished to assess the contribution of $K^+$ conductances to the extra TPM-induced outward currents; we therefore recorded with 3 M Cs acetate-filled microelectrodes, in an attempt to minimize the outward current induced by positive voltage jumps (Galvan *et al*., 1985), and to hopefully dissect the underlying mechanism of the sAHP enhancement by TPM. The experiments were routinely conducted in 1 $\mu$M TTX to block $Na^+$ conductances. In 25 Cs acetate-loaded cells (at least 30 min loading period), the directly evoked action potential under current clamp (Figure 7a) showed a characteristically prolonged plateau phase, due to activation of high voltage-activated (HVA) $Ca^{2+}$ conductances (Galvan *et al*., 1985), followed by a prominent slow depolarizing after potential (DAP). Under voltage clamp, the DAP is manifested as a slow inward tail current following the offset of the depolarizing command (Constanti *et al*., 1985) and is most likely mediated by a $Ca^{2+}$-activated nonselective (CAN), cation conductance (Kang *et al*., 1998). Despite $Cs^+$ loading, TPM still produced a significant enhancement of the *residual* outward current relaxation (mean amplitude = 1.84 ± 0.48 nA vs 1.56 ± 0.42 nA control; $P<0.001$), but now instead of an outward $sI_{AHP}$ tail, an *enhanced* slow *inward* after-tail current was revealed (124 ± 47 pA amplitude, 7.52 ± 1.15 s duration vs 114 ± 52 pA amplitude, 3.18 ± 0.32 s duration: control) (means significantly different from control; $P<0.001$; paired *t*-tests), most likely representing a mixture of decaying (enhanced) HVA $Ca^{2+}$ current and CAN current. Accordingly, both the TPM-induced outward relaxation component and the slow inward tail current were eliminated by further adding $Cd^{2+}$ (200 $\mu$M; $n=17$), indicating their dependence on external $Ca^{2+}$ entry (Figure 7b).

One obvious possibility was that this enhanced HVA $Ca^{2+}$ current component of the inward after-tail current revealed by $Cs^+$ loading was mediated *via* L-type $Ca^{2+}$ channels. Indeed, in the presence of the selective L-type $Ca^{2+}$ channel blocker nifedipine (20 $\mu$M; $n=5$) (Bean, 1989), the TPM-induced tail was blocked, suggesting that L-channels were providing the $Ca^{2+}$ influx for its generation (Figure 8a). Nifedipine also consistently abolished the 'plateau' region that was characteristically present in the TPM *I–V* plot between ~−55 and −35 mV in $Cs^+$-loaded cells, and reduced the TPM-induced extra outward rectification at more positive potentials, resulting in an unusual crossover of the curves at around −35 mV (Figure 8b); the mean potential at which crossover of

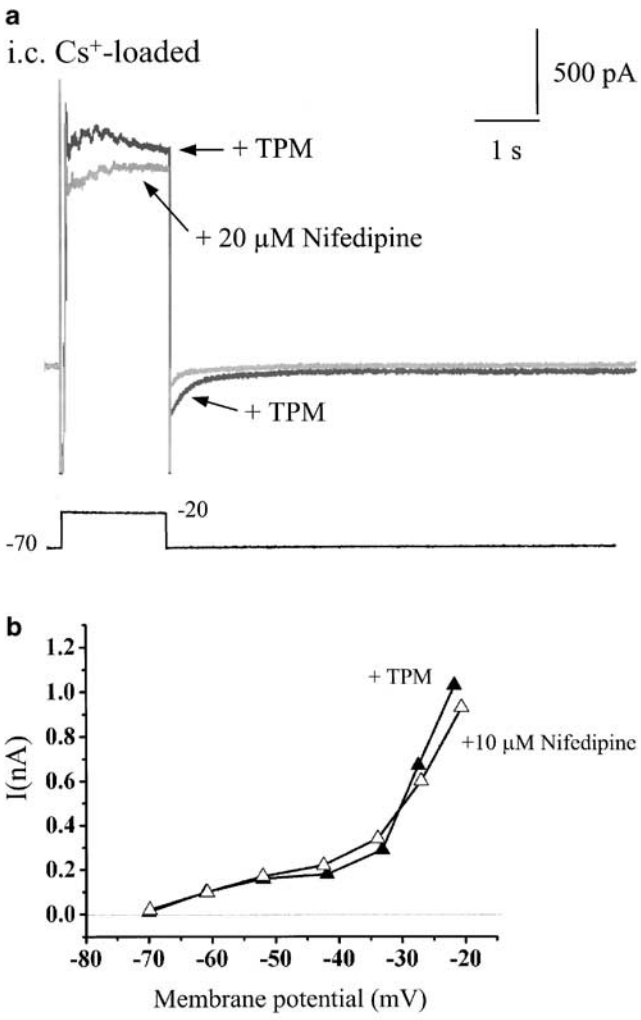


**Figure 8** TPM-induced after-tail current is blocked by the L-type $Ca^{2+}$ channel blocker nifedipine. (a) Superimposed clamp currents (in TTX) in response to a +50 mV (1.5 s) voltage jump recorded in a $Cs^+$-loaded neurone held at −70 mV. In the presence of 20 $\mu$M TPM, an enhanced outward current and *inward* after-tail current are induced, both of which are reduced by 20 $\mu$M nifedipine (10 min) (note, TPM generated a negligible (0.05 nA) steady outward current shift in this cell). (b) Corresponding steady-state *I–V* relation in TPM showing abolition by nifedipine of the characteristic 'plateau' region present between −55 and −35 mV, and reduction of extra TPM-induced outward rectification at more positive potentials; note the curious crossover of *I–V* plots at around −35 mV, suggesting an interplay between enhanced inward and residual outward-rectifying current components.

the *I–V* plots occurred was $-30.6 \pm 0.9$ mV ($n = 5$). This result suggests a possible interplay between enhanced inward and residual outward rectifying components in $Cs^+$-loaded cells in the presence of TPM.

It is worth noting that in 17/25 $Cs^+$-loaded cells, TPM (20 $\mu$M) still evoked a slow outward shift in the baseline holding current, although this was significantly smaller (mean $= 0.17 \pm 0.05$ nA; $P < 0.05$) compared with cells recorded with normal K acetate electrodes ($0.36 \pm 0.26$ nA, $n = 32$). Interestingly, in three cells tested, $Cs^+$ loading abolished outward current responses induced by 20 $\mu$M baclofen under voltage clamp, despite an incomplete blockade of the outward current induced by TPM in the same neurone (mean $= 0.18 \pm 0.07$ nA); this suggested that an effective equilibration with $Cs^+$ was occurring under our conditions. In 8/25 $Cs^+$-loading experiments, no TPM outward current was generated (compare with 2/34 instances where TPM failed to evoke slow outward current in normally recorded cells under voltage clamp). This provides further evidence that the slow TPM-induced current was predominantly mediated by $K^+$.

### *Does PKA phosphorylation modulate the effect of TPM on the sAHP?*

In view of the 'unifying' hypothesis proposed by Shank *et al.* (2000) that TPM prefers to interact with *de*-phosphorylated target ion channels/drug receptors, we initially wished to investigate whether PKA-mediated phosphorylation would modulate this action of TPM on the sAHP. According to the hypothesis, such a prephosphorylation of L-type $Ca^{2+}$ channels should *occlude* the action of TPM. In the representative experiment of Figure 9, a control sAHP under current clamp and the corresponding outward s$I_{AHP}$ tail current under 'hybrid' voltage clamp were initially recorded in control solution (Figure 9a, b, respectively). On adding forskolin (20 $\mu$M; 15 min), a direct activator of adenylate cyclase, and ultimately PKA (Simonds, 1999), the sAHP and s$I_{AHP}$ were abolished and replaced by a small slow afterdepolarization (sADP) (mean $= 4.8 \pm 0.96$ mV) and a small $I_{ADP}$ tail ($95 \pm 34$ pA; $n = 4$), most likely due to a rise in intracellular cAMP and direct inhibitory action on the $Ca^{2+}$-activated $K^+$ current generation mechanism, as previously reported for hippocampal neurones (Nicoll, 1988; Knöpfel *et al.*, 1990). Accordingly, forskolin also significantly reduced spike accommodation, as was evident from the increased number of spikes fired during the depolarizing stimulus (means: $38 \pm 9$ spikes, control; $60 \pm 4$ spikes in forskolin; $P < 0.05$, $n = 4$) (Figure 9a). On further addition of TPM, this prior effect of forskolin did *not* prevent the reappearance of the characteristically prolonged sAHP and corresponding s$I_{AHP}$ tail (mean $= 580 \pm 140$ pA amplitude, $9.72 \pm 4.34$ s duration), suggesting that PKA-dependent phosphorylation mechanisms were *not* occluding the action of TPM on L-channels, or possibly also on the slow $Ca^{2+}$-activated $K^+$ channels (most likely non-SK-BK channels) themselves (Sah & Faber, 2002). Spike firing was reduced in forskolin + TPM (mean $= 44 \pm 10$ spikes), but the mean values were not significantly different from forskolin alone ($P > 0.5$, $n = 4$). Similar end results were obtained in four other cells, where the converse experiment was carried out (not shown): here, TPM was applied *first* to induce a characteristic sAHP enhancement, then forskolin added in combination. Under this condition, a further prolongation of the sAHP and s$I_{AHP}$ tail ($570 \pm 121$ pA amplitude, $9.27 \pm 3.94$ s duration) appeared, most likely due to forskolin's own recognized effect in enhancing the activity of the underlying L-channels (Anwyl, 1991; Dolphin, 1999), which was then supplementing (or possibly synergizing with) that of TPMs. Interestingly, forskolin did not significantly affect spike accommodation in the presence of TPM (mean number of spikes $= 28 \pm 4$ in TPM vs $25 \pm 3$ in TPM + forskolin, $P > 0.5$; however, both means were significantly different from control: $46 \pm 5$, $P < 0.05$; $n = 4$). Either way, it is clear that TPM can apparently override the inhibitory effect of forskolin on the sAHP and spike

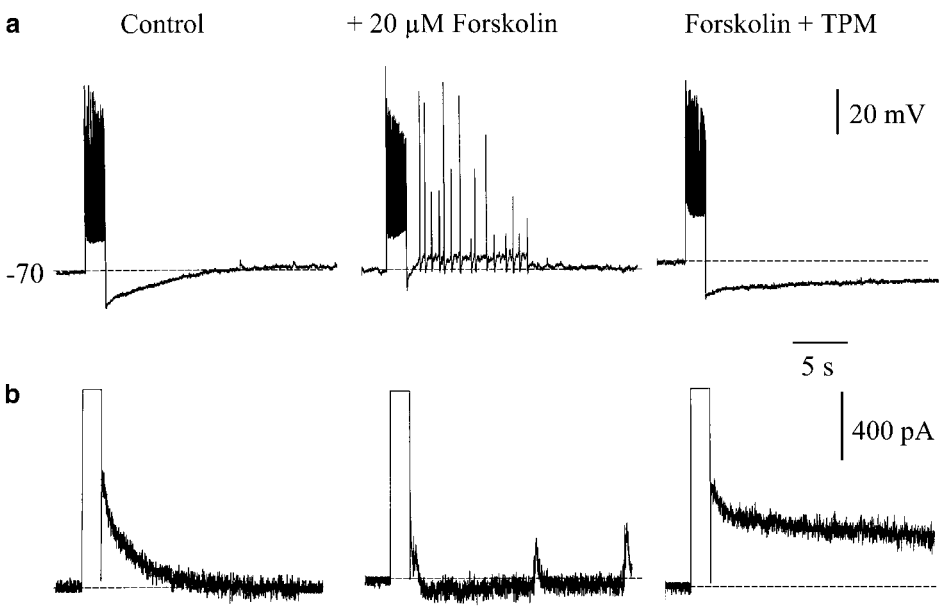


**Figure 9** PKA-mediated phosphorylation does not occlude the action of TPM on the sAHP. (a) Neurone maintained at −70 mV membrane potential. A prolonged depolarizing stimulus (1.5 s, +2.0 nA) induces a poststimulus sAHP in control solution, which is abolished (converted to a sADP with superimposed repetitive firing) in the presence of the direct adenylate cyclase activator forskolin (20 $\mu$M; 15 min) applied alone. On further adding, TPM (20 $\mu$M, 10 min), a prolonged sAHP tail, re-appeared. (b) Corresponding s$I_{AHP}$ tail currents recorded under 'hybrid' voltage clamp at −70 mV. Traces show currents in control solution, after blockade by forskolin (middle) and after adding TPM; note the re-appearance of s$I_{AHP}$ tail, with prolonged decay time. An outward baseline shift of 0.42 nA induced by TPM was omitted for clarity.

accommodation caused by intracellular PKA-dependent phosphorylation.

### *TPM effects are mimicked by carbonic anhydrase (CA) inhibitors*

TPM is known to have a CA inhibitory action, although its potency in this respect appears to be controversial. In earlier studies, the $K_i$ value for TPM vs the physiologically important isozyme CA II was found to be in the micromolar range, and at least an order of magnitude less than that of the standard membrane-permeant CA inhibitor ACTZ (Dodgson *et al.*, 2000). More recently, Casini *et al.* (2003) concluded that TPM was in fact a very potent CA II inhibitor, with nanomolar affinity comparable to that of ACTZ. ACTZ has long been recognized for its antiepileptic properties (Millichap *et al.*, 1955), and is still occasionally used today as adjunctive therapy for the management of partial refractory seizures (Brodie, 2001) and absences (Panayiotopoulos, 2001); its precise mechanism of anticonvulsant action, however, has never been fully established, although it is generally believed to involve CA inhibition, thereby promoting changes in intracellular and extracellular pH and modulating neuronal excitability indirectly (Resor *et al.*, 1995; see also Discussion). We wished to examine whether the observed membrane effects of TPM on olfactory neurones could be partly (or wholly) explained by such changes in $pH_o$ (and/or $pH_i$), caused as a consequence of CA inhibition, and to what extent these effects could be mimicked by ACTZ (or other CA inhibitors).

In our experiments, ACTZ (20 $\mu$M, 10 min; $n=8$) regularly elicited a slow, reversible membrane hyperpolarization (mean = 9 ± 2 mV, significantly different from rest level; $P<0.001$) and a decrease in input resistance (mean = 24 ± 8%; $P<0.001$) similar to those elicited by 20 $\mu$M TPM (cf. also Herrero *et al.*, 2002). Under voltage clamp, ACTZ evoked a slow outward current shift (mean = 0.33 ± 0.17 nA; $n=8$) with similar amplitude and onset to the TPM-evoked current (cf. Figure 2a), but with slower offset on drug washout. An example of an experiment where the membrane effects of TPM and ACTZ were compared under voltage clamp at –70 mV holding potential is shown in Figure 10. TPM (20 $\mu$M) and ACTZ (20 $\mu$M) (10 min applications) produced similar-amplitude slow outward currents when recorded in a single neurone (Figure 10a), and, when TPM was superimposed at the peak of the ACTZ current, the effect of TPM was occluded, in accordance with the current-clamp findings of Herrero *et al.* (2002) on hippocampal cells. The converse also applied; thus, in the experiment of Figure 10b, an initial application of ACTZ (20 $\mu$M, 10 min) produced an outward current response, followed by a typical TPM response. In the presence of TPM, the effect of the same dose of ACTZ was also clearly occluded; this would confirm that both TPM and ACTZ were sharing a common mechanism of action in this respect, although whether this involved CA inhibition was still unclear.

The possible role of CA inhibition in explaining the enhancing effects of TPM and on the sAHP was addressed in a second series of tests, as illustrated in Figure 10c, d. Here, a control burst of action potentials with ensuing sAHP (Figure 10c) and the corresponding s$I_{AHP}$ tail current under hybrid voltage clamp (Figure 10d) were initially recorded in control medium. Like TPM, 20 $\mu$M ACTZ was also able to significantly enhance (~25%) (281 ± 122 pA control vs 351 ± 117 pA in ACTZ; $P<0.001$) and prolong the duration (~77%) (3.56 ± 0.83 vs 6.30 ± 0.9 s in ACTZ $P<0.001$) ($n=8$) of the s$I_{AHP}$ tail current underlying the sAHP (cf. Figure 5); however, in contrast with the occlusive slow outward current responses,

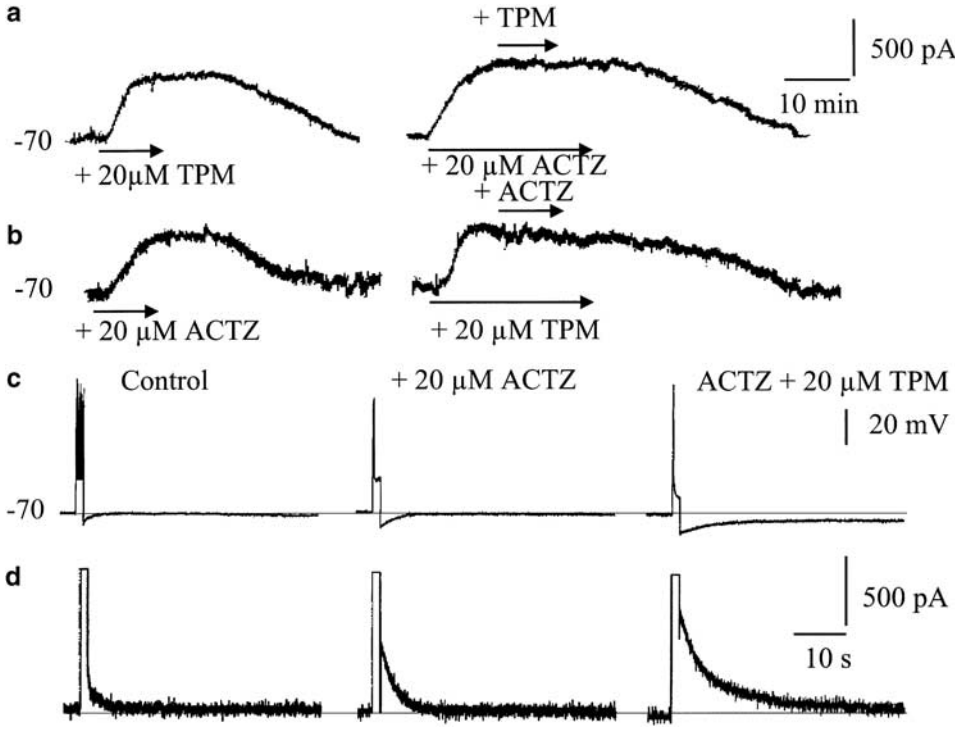


**Figure 10** The carbonic anhydrase inhibitor ACTZ (10 $\mu$M) mimics and occludes the neuronal effects of TPM. (a, b) Comparison of slow outward membrane currents (recorded under voltage clamp at $-70$ mV) produced by TPM and ACTZ, respectively, in different neurones (10 min applications). (a) Control response to TPM (20 $\mu$M) was obtained initially, followed by 30 min recovery. ACTZ (20 $\mu$M; 25 min) applied to the same cell evoked a comparable response and occluded the effect of TPM coapplied at the peak of the ACTZ current. (b) Initial response to ACTZ was followed by a similar response to TPM, which occluded the effect of ACTZ coapplied at the peak. (c) sAHP recorded in control Krebs solution, then after 10 min in ACTZ; note the TPM-like enhancement and prolongation of sAHP, along with increased spike accommodation during depolarizing pulse (cf. Figure 5). TPM (20 $\mu$M) was then applied to the same cell (in ACTZ), producing a *further* comparable enhancement and prolongation of the sAHP (membrane potential corrected to $-70$ mV throughout by injection of steady positive current). (d) Corresponding tail currents recorded under 'hybrid' voltage clamp at $-70$ mV show clear enhancement of the s$I_{AHP}$ tail in the presence of ACTZ alone or ACTZ plus TPM, respectively. The set current stimulus was + 2.0 nA, 1.5 s throughout. An outward baseline shift of 0.30 nA induced by ACTZ was omitted for clarity; TPM did not induce any further outward current in the presence of ACTZ.

on coapplication with ACTZ, TPM (20 $\mu$M) still produced a *further* clear enhancement and prolongation of the sAHP and s$I_{AHP}$ tail current ($351 \pm 117$ pA amplitude, $6.30 \pm 0.9$ s duration in ACTZ vs $523 \pm 124$ pA, $8.24 \pm 1.7$ s in ACTZ + TPM; *t*-tests: $P < 0.001$), suggesting an additive or possible synergistic effect of these two agents in evoking this response, and which could only really be explained if their effects were *independent* of any CA inhibitory activity.

A crucial question that remained was whether the abilities of TPM and ACTZ to generate a slow outward current and enhancement/prolongation of the sAHP (along with their presumed actions on CA, and membrane consequences thereof) were mediated intracellularly or extracellularly (or both). To address this, we tested the effects of the hydrophilic CA inhibitor benzolamide (CL 11366; BZ), which is generally considered to be poorly membrane permeant (Maren, 1977; Leniger *et al*., 2002). Surprisingly, BZ mimicked all of the observed effects of TPM (and ACTZ), although with less potency. Thus, bath application of BZ (50 $\mu$M, 10 min) induced a slow membrane hyperpolarization (mean $= 7 \pm 2$ mV; $n = 7$) accompanied by decrease in membrane input resistance (mean $= 18 \pm 8\%$; $n = 7$), comparable to the responses elicited by TPM or ACTZ. Under voltage clamp, BZ evoked a slow outward current shift (mean $= 0.24 \pm 0.03$ nA; $n = 7$) with similar amplitude and onset/offset as the TPM- or ACTZ-evoked currents (not shown). Accordingly, when TPM (20 $\mu$M, $n = 3$) or ACTZ (20 $\mu$M, $n = 3$) were applied in the presence of BZ, their usual outward current-inducing effects were occluded. Benzolamide also had a small enhancing/prolonging effect on the sAHP and the underlying s$I_{AHP}$ tail current ($\sim 16\%$ enhancement) (control mean $= 278 \pm 129$ vs $324 \pm 131$ pA in BZ; $P < 0.001$) ($\sim 72\%$ prolongation) ($3.59 \pm 0.89$ s control vs $6.16 \pm 0.86$ s in BZ; $P < 0.001$; paired *t*-tests; $n = 7$), and, moreover, a further coapplication of TPM produced its typical additive effect on the outward current tail ($324 \pm 131$ pA amplitude, $6.16 \pm 0.86$ s duration in BZ vs $562 \pm 117$ pA, $7.93 \pm 1.4$ s in BZ + TPM; *t*-tests: $P < 0.001$). These important results with BZ strongly suggest that all of our observed TPM (and ACTZ)-induced effects could be mediated *extracellularly via* an interaction with the outer part of the neuronal cell membrane; however, could this mechanism still involve CA inhibition and extracellular/intracellular pH changes?

### *Effect of switching to HEPES-buffered (bicarbonate-free) bathing medium*

As a decisive further test of whether the common effects elicited by TPM and ACTZ were CA-mediated and therefore due to a change in $pH_i$ and/or $pH_o$, we performed a set of

experiments ($n=10$ cells) in which the normal bicarbonate-buffered Krebs bathing medium (bubbled with $O_2/CO_2$) was substituted by a solution containing HEPES (at the same $pH_o$) instead of $NaHCO_3$ and bubbled with pure $O_2$ (Church, 1992). Under these conditions, the activity of CA would be blocked, due to the absence of its natural substrates. Neurones were first impaled in the normal Krebs medium and a control slow outward current response to TPM or ACTZ recorded under voltage clamp (depending on the drug to be subsequently applied); a switch was then made to the $HCO_3^-/CO_2$-free HEPES solution and a second drug response obtained after 30 min incubation. On first applying HEPES-buffered medium, there was an initial depolarization of $\sim$2–3 mV (mean $=2.4\pm0.9$ mV) within 10 min, accompanied by a stable decrease in the input resistance (mean $=8.1\pm2.3\%$), followed by a small hyperpolarization (mean $=3.2\pm0.6$ mV) within the next 10–15 min, which was maintained after 30 min (Figure 11a) (cf. Church, 1992).

In HEPES medium under voltage clamp at $-70$ mV, application of either TPM ($n=6$) or ACTZ ($n=4$) *still* elicited slow outward currents of comparable time course and amplitude to those measured in control Krebs solution (Figure 11b, c) (the mean TPM current $=0.38\pm0.04$ nA control vs $0.40\pm0.04$ nA in HEPES; mean ACTZ current $=0.35\pm0.03$ nA control vs $0.34\pm0.03$ nA in HEPES). Both means in HEPES were not significantly different from control values; *t*-tests, $P>0.5$). This confirms that the common outward currents generated by the two drugs (and most likely also by BZ) are not mediated by CA inhibition, but they probably share a common site of action on the outside of the cell membrane. However, we cannot exclude the possibility that, under normal conditions, the CA-inhibitory properties of these compounds (and consequent changes in neuronal $pH_i$/$pH_o$) might also somehow contribute to their observed anticonvulsant activity.

Finally, we also examined whether switching to a HEPES-buffered medium would affect the ability of TPM to modulate the s$I_{AHP}$ tail current. In six neurones studied under hybrid voltage clamp, the s$I_{AHP}$ tail was significantly reduced in amplitude after 30 min exposure to HEPES solution ($\sim$48%) ($413\pm48$ pA control vs $213\pm42$ pA in HEPES; $P<0.005$). A similar effect was reported on the sAHP in hippocampal neurones, and is likely to be due to an acidic shift in cytoplasmic pH, affecting the $Ca^{2+}$-activated $K^+$ conductance mechanism directly (Church, 1992). Despite this suppression, application of 20 $\mu$M TPM in HEPES medium still produced a significant enhancement ($\sim$83%) ($390\pm54$ pA vs HEPES control; $P<0.002$) and increase in duration ($\sim$78%) ($3.42\pm0.63$ vs $1.91\pm0.47$ s HEPES control; $P<0.002$) of the s$I_{AHP}$, suggesting that this second novel effect of TPM was also not mediated by CA inhibition.

## Discussion

In the present study, we found that TPM, applied at therapeutically effective concentrations, produced two main effects on olfactory cortical neurones: (1) a slow, dose-

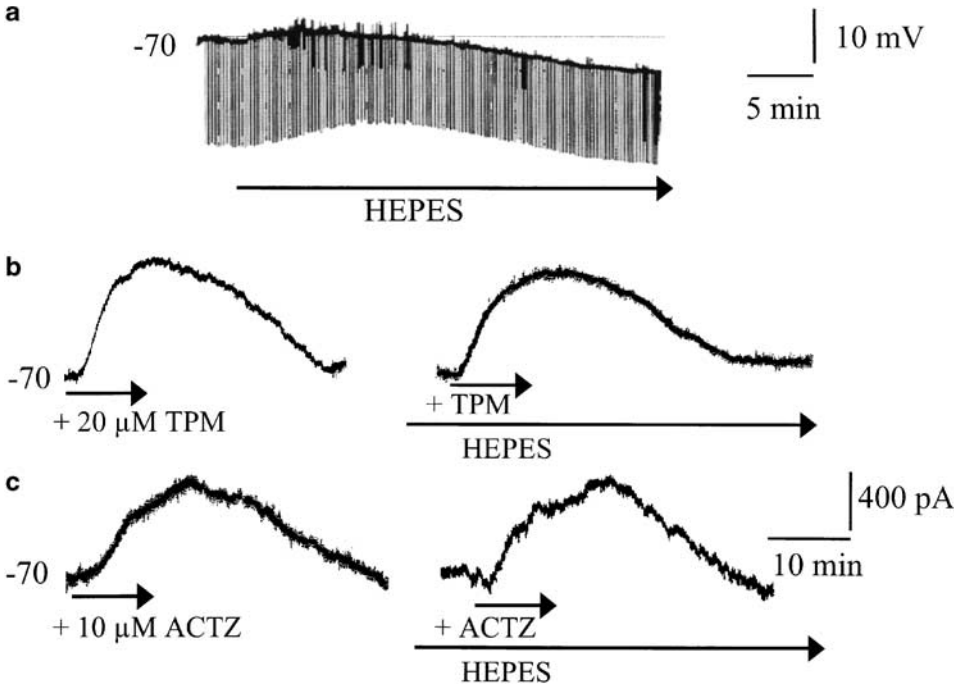


**Figure 11** TPM and ACTZ effects on outward current generation are unaffected by recording in HEPES-buffered bathing medium. (a) Slow-speed chart record showing the effect of switching from normal bicarbonate-buffered Krebs solution, to bicarbonate/$CO_2$-free HEPES-buffered solution on membrane potential and input resistance of a neurone maintained at $-70$ mV membrane potential by positive current injection. Downward deflections represent hyperpolarizing electrotonic potentials evoked by regular current pulses ($-0.5$ nA) injected every 1.5 s throughout; note the small early depolarization, transient decrease in resistance, and subsequent sustained hyperpolarization in HEPES medium. (b, c) Slow outward currents induced by TPM (20 $\mu$M, 10 min) or ACTZ (20 $\mu$M, 10 min) under voltage clamp at $-70$ mV (followed by slow recoveries on washout), recorded initially in normal Krebs solution (left) and then after 30 min exposure to HEPES medium (right). Note, both responses are essentially unaltered in the HEPES-buffered solution, and therefore not dependent on carbonic anhydrase inhibition for their generation.

dependent and reversible membrane hyperpolarization, accompanied by a decrease in membrane resistance and inhibition of repetitive action potential firing, and (2) an enhancement and prolongation of a poststimulus sAHP that follows a burst of action potentials; the latter phenomenon has not previously been reported for TPM on other neurone types (Hanaya *et al*., 1998; Kawasaki *et al*., 1998; DeLorenzo *et al*., 2000; Jahromi *et al*., 2000; Herrero *et al*., 2002) and so may well be specific to cortical cells. Interestingly, the mechanisms responsible for the slow hyperpolarization and sAHP enhancement/prolongation effect appear to be distinct, since, in a minority of cells where the sAHP was not enhanced by TPM, the slow hyperpolarization response was still generated.

### *TPM-induced membrane hyperpolarization and slow outward (clamp) current*

Under voltage clamp, the TPM-induced hyperpolarization was manifested as a slowly developing outward membrane current and increase in membrane conductance, most likely carried largely (but not exclusively) by $K^+$ ions, that was slowly reversible on drug washout. Similar hyperpolarizing effects of TPM were recorded by Kawasaki *et al*. (1998) in subicular neurones, and also by Herrero *et al*. (2002) in hippocampal neurones, albeit at higher concentrations of TPM (200 and 100 $\mu$M, respectively) than used in the present study. In the former case, the TPM hyperpolarization was abolished by bicuculline, and therefore was attributed to an indirect increase in $GABA_A$ receptor function (White *et al*., 2000), while, in the latter, the hyperpolarizing response reversed near the $K^+$ equilibrium potential ($E_K$) and was abolished by the general $K^+$ channel blocker $Ba^{2+}$ (Eaton & Brodwick, 1980), suggesting an exclusive increase in a $K^+$ conductance. In our experiments, the TPM-induced slow outward current was unaffected by bicuculline, and therefore was not mediated by $GABA_A$ receptors; also, the TPM current, although reduced in amplitude (suggesting it was mainly $K^+$-mediated), was incompletely blocked by 1 mM $Ba^{2+}$, and (unlike the baclofen-induced current) failed to show a clear reversal at negative membrane potentials, indicating that other membrane mechanisms or ionic conductances (yet to be identified) may be involved in its mediation. Alternatively, the apparent $Ba^{2+}$ insensitivity of the postulated outward $K^+$ current activated by TPM could be a feature of the $K^+$ channel itself, rather than an indication that another conductance mechanism is involved. Indeed, there is evidence that some neuronal $K^+$ channels may be partially sensitive (Krapivinsky *et al*., 1998) or completely insensitive (Wagner & Dekin, 1993) to blockade by high concentrations of this cation.

Neuronal membrane hyperpolarizations showing lack of reversal at $E_K$, can be produced by a complex combination of ionic conductance changes coupled to possible changes in hyperpolarizing electrogenic pump activity, and are not unprecedented in the literature (see Trotier & Doving, 1996; estimated reversal potential of $Na^+$ pump current = −143 mV); such hyperpolarizations may or may not be associated with a small increase in membrane conductance (Thompson & Prince, 1986; Mimura *et al*., 1994; Parker *et al*., 1996; Marinelli *et al*., 2000). In our case, involvement of the conventional $Na^+$–$K^+$–ATPase pump was unlikely, since ouabain was ineffective against the TPM current (cf. Gustafsson & Wigström, 1983). Other electrogenic pump mechanisms triggered by TPM with reversal levels outside the normal physiologic range could conceivably generate an outward current, for example, a $K^+$-dependent $Na^+$–$Ca^{2+}$ exchanger (NCKX2) operating in reverse mode (Dong *et al*., 2001); however, such a process might inevitably be neurotoxic, due to a rise in cytosolic $Ca^{2+}$.

Another possibility is that the unusual behaviour of the TPM current was due to activation by TPM of a novel $K^+$ conductance that is de-activated at potentials negative to −80 mV; the result of experiments of the type illustrated in Figures 2a, b would certainly support such a hypothesis. Alternatively, lack of reversal of the TPM outward current with hyperpolarization could be due to a simultaneous increase in $K^+$ conductance and a *decrease* in a persistent cationic conductance (TTX-resistant) with a more positive reversal level, or may reflect the fact that it is generated at a site(s) remote from the somatic recording electrode, with a contribution of electrotonic dendritic coupling, as appears to occur following opioid peptide application in locus coeruleus neurones (Alreja & Aghajanian, 1993; Travagli *et al*., 1995; Ishimatsu & Williams, 1996). Further detailed experiments would be required to confirm such complex mechanisms in our cells.

The fact that $Cs^+$ loading only partially reduced the TPM slow outward current could be interpreted in several ways; the most parsimonious explanation is that $Cs^+$ blocked the proposed $K^+$ component of the TPM current, leaving the $Ba^{2+}$-resistant (cationic) component. Alternatively, a component of the proposed TPM outward $K^+$ current may have flowed through $Cs^+$-permeable $K^+$ channels (cf. Wigmore & Lacey, 2000). Our present data do not allow us to distinguish between these two possible mechanisms.

Hyperpolarization and reduction in neuronal input resistance *via* activation of a $K^+$ conductance is a rather novel mechanism of anticonvulsant action that is not shared by other conventional AEDs; such a mechanism would be expected to reduce neuronal excitability and therefore prevent seizure generation and spread in a highly effective manner. Future development of new drugs that activate 'background' $K^+$ channels regulating neuronal cell excitability and resting potential could indeed prove therapeutically useful as novel anticonvulsants (Wickenden, 2002). At present, only retigabine that opens KCNQ $K^+$ channels, thereby modulating the activity of the M-type $K^+$ current ($I_M$), is known to exert its anticonvulsant action *via* this manner (Main *et al*., 2000). Similar direct activation of M channels by TPM in our experiments would certainly explain the $Ba^{2+}$-sensitive component of the outward current response, and cannot presently be excluded. However, the possibility that the TPM current flows through $Ca^{2+}$-activated $K^+$ channels (cf. Tricarico *et al*., 2000) was unlikely, since the response was unaffected by the general $Ca^{2+}$ channel blocker $Cd^{2+}$. The exact mechanism of the TPM-induced hyperpolarizing response in cortical neurones, therefore, still remains to be determined (see also below).

### *TPM enhances and prolongs the sAHP by modulating an L-type $Ca^{2+}$ current*

The novel enhancement and dramatic prolongation of the sAHP by TPM was found to be dose-dependent, reversible, and (unlike the slow hyperpolarizing response) reliant on $Ca^{2+}$

entry, as it was blocked by external $Cd^{2+}$. Two possibilities could account for this phenomenon: an increase in $Ca^{2+}$ entry and/or a direct enhancement in activity of the underlying $Ca^{2+}$-activated $K^+$ channels themselves. Intracellular loading with the $Ca^{2+}$ chelators EGTA or BAPTA in an attempt to eliminate the $Ca^{2+}$-dependent $K^+$ conductance, and therefore confirm the role of $Ca^{2+}$ in generating the extra TPM-induced outward current relaxation under voltage clamp (Figure 6), was not attempted in our study, since data obtained in our own laboratory (Constanti *et al*., 1993) and those of others (Schwindt *et al*., 1992, Zhang *et al*., 1995; Jahromi *et al*., 1999; Velumian & Carlen, 1999) have shown that 'sharp' or patch-clamp intracellular recording with EGTA or BAPTA-filled electrodes in cortical or hippocampal neurones can lead to a paradoxical *potentiation* and *prolongation* (rather that elimination) of the sAHP; this curious effect is apparently due to a prolongation of the intracellular $Ca^{2+}$ signal following stimulus-induced $Ca^{2+}$ entry into the cell, and is reliant on an interplay between the intracellular $Ca^{2+}$ rise, normal $Ca^{2+}$ extrusion/sequestration and the $Ca^{2+}$ buffering capacity/binding kinetics of these mobile buffers. Alternative application of the cell membrane-permeable buffer analogue BAPTA-AM also failed to reliably influence the sAHP in our neurones, as previously reported (Constanti *et al*., 1993). Clearly, such properties of these chelators would complicate rather than clarify any intrinsic effects of TPM on sAHP generation. Likewise, in view of the findings of Lancaster & Batchelor (2000), who showed that intracellular loading with BAPTA (*via* whole-cell recordings) could *itself* induce a large steady-state outward $K^+$ current in rat hippocampal CA1 neurones by directly activating the same $Ca^{2+}$-activated $K^+$ channels that underlie the sAHP, we also consider it unlikely that such loading experiments would help in resolving the issue of possible $Ca^{2+}$ mobilization by TPM in generating the slow (hyperpolarizing) outward current shift (see Results above).

Although $Cs^+$ loading alone did not completely suppress outward current relaxations induced by positive voltage jumps (indicating the presence of $Cs^+$-permeable $K^+$ channels; cf. Wigmore & Lacey, 2000), the experiments were diagnostic in revealing an underlying long poststimulus inward tail current, that was itself enhanced and prolonged by TPM. The fact that this induced tail current was blocked by the specific L-channel antagonist nifedipine (Bean, 1989) indicates that an L-type $Ca^{2+}$ current (activated during the depolarizing stimulus) was the main source of $Ca^{2+}$ entry and the most likely target of action of TPM in our neurones. Therefore, the observed enhancement of the sAHP by TPM might be largely, if not entirely, explained by the *enhancement* of an underlying L-type $Ca^{2+}$ current, although a direct enhancing effect on the $Ca^{2+}$-activated $K^+$ channels cannot be entirely excluded. An analogous effect has been reported in neostriatal neurones following application of the L-channel agonist BayK 8644; Hernandez-Lopez *et al*., 1996). This result contrasts with the recent findings of Zhang *et al*. (2000), who observed a *block* of L-currents by TPM (at 10 $\mu$M, but *not* at 50 $\mu$M) in rat dentate granule cells. Their data strongly suggest there may be a two-fold effect of TPM on L-currents, with depression being evident at low concentrations, and perhaps a combination of depression and enhancement at higher doses. Interestingly, they also found that *non*-L-type currents in these cells were transiently *increased* by TPM at high concentrations (50 $\mu$M). In our experiments, we only ever observed enhancement of the sAHP by 20 or 50 $\mu$M TPM (see above), and the slow inward current tail revealed after $Cs^+$ loading was consistently enhanced by 20 $\mu$M TPM.

Regarding the phosphorylation hypothesis of Shank *et al*. (2000), we found that enhancement and prolongation of the sAHP, and underlying nifedipine-sensitive slow inward tail current by TPM, did not appear to involve an interaction of this drug at PKA-dependent phosphorylation sites, since it still occurred in the presence of the direct adenylate cyclase activator forskolin (Simonds, 1999), which itself depressed the sAHP; however, an interaction of TPM at *other* potential phosphorylation sites on the L-channel (and possibly also the $Ca^{2+}$-activated $K^+$ channel itself) cannot be discounted. The fact that TPM could apparently override the inhibitory effect of a rise in intracellular cAMP produced by forskolin on the sAHP was indeed interesting, and could well be relevant for its antiepileptic action, since it could overcome the effect of many neurotransmitters (e.g. noradrenaline, 5HT, histamine) that would normally increase neuronal cell excitability by raising intracellular cAMP *via* a PKA-dependent pathway (Pedarzani & Storm, 1993). To our knowledge, TPM itself is not an effective inhibitor of PKA at doses covering the therapeutic range (1–100 $\mu$M; R.P. Shank, Johnson & Johnson Pharmaceutical Research and Development, L.L.C., PA, U.S.A.; personal communication); therefore, its enhancing effect on the sAHP (and apparent reversal of forskolin action) was unlikely to involve a change in basal phosphorylation–dephosphorylation turnover (Pedarzani *et al*., 1998) and must be taking place *via* a PKA-independent mechanism. If phosphorylation by forskolin enhances the underlying L currents (Anwyl, 1991; Dolphin, 1999) and also inhibits the sAHP directly, why does the latter effect predominate when forskolin alone is applied (Figure 9)? Perhaps, it is a question of balance between two opposing effects (i.e. functional antagonism). Under normal conditions, the sAHP inhibitory action of forskolin may be the stronger effect (with some 'spare' capacity). In TPM, $Ca^{2+}$ entry *via* L channels may be so enhanced that it overrides the inhibitory effect, so only sAHP enhancement is seen.

### *Are the effects of TPM mediated by CA inhibition?*

Several reports have appeared showing that CA inhibition (presumably of both intracellular and interstitial isoforms of the enzyme; cf. Pasternack *et al*., 1993; Tong *et al*., 2000) by the 'classical' CA inhibitor ACTZ can induce external neuronal acidification (Vorstrup *et al*., 1989; Meierkord *et al*., 2000), most likely due to $CO_2$ accumulation and interference with $HCO_3^-$ availability, and therefore a decrease in the efficiency of the $CO_2/HCO_3^-$ buffering system to handle extracellular metabolic acid shifts (due to $H^+$ extrusion and/or lactic acid generation). Extracellular acidification is also likely to be mirrored by a comparable reduction in $pH_i$ *via* an unknown mechanism (Church *et al*., 1998). If TPM and ACTZ were producing a similar extracellular/intracellular acidification *via* CA inhibition, then could this explain their observed generation of a slow hyperpolarization and sAHP enhancement in olfactory cortical neurones? Changes in $pH_o/pH_i$ are known to affect intrinsic neuronal membrane properties, including outward- and inward-rectifying $K^+$ currents (Tombaugh & Somjen, 1996; Zhu *et al*., 1999), $Ca^{2+}$ currents (Tombaugh & Somjen, 1997),

and other $Ca^{2+}$-dependent conductances (Church *et al*., 1998; Church, 1999). It is now clear that decreasing $pH_o$ is anticonvulsant, causing neuronal *de*polarization, a decrease in input resistance while *depressing* burst firing, sAHP amplitude and HVA $Ca^{2+}$ currents; the opposite applies for an increase in $pH_o$ (Church & McLennan, 1989; Church, 1992; 1999).

Herrero *et al*. (2002) showed that ACTZ elicited slow hyperpolarizing effects similar to those of TPM on hippocampal cells; in particular, it occluded the TPM-evoked hyperpolarization. We obtained similar results with ACTZ and TPM under voltage clamp in our system, and moreover showed that benzolamide (BZ), a poorly –membrane-permeant CA inhibitor, was also capable of evoking an outward current shift and occlusion of the effect of TPM. Herrero *et al*. (2002) suggested that both TPM and ACTZ may activate a common $K^+$ conductance by virtue of their effects on interstitial (and intracellular?) CA activity. A fast inward rectifier (Kir2.3) channel or background 'leak' TASK-like $K^+$ channel (Lesage, 2003) were suggested as likely candidates, both channels being capable of sensing $pH_o$ changes at near physiological levels; both channels, however, are *blocked* rather than opened by external acidification (Zhu *et al*., 1999; Bayliss *et al*., 2001); thus, an alternative channel type(s) must be involved. Our experiments confirmed that similar slow outward currents could be produced by TPM and two other CA inhibitors possessing the sulphonamide moiety; therefore they were all presumably acting *via* a common mechanism that could possibly have involved alterations in intracellular and/or extracellular pH consequent to CA inhibition (see below). However, since the outward currents induced by TPM or ACTZ were unaffected in a bicarbonate/$CO_2$-free HEPES-buffered bathing medium, the mechanism of generation could not have involved CA inhibition, as implied by the data of Herrero *et al*. (2002).

Since TPM and ACTZ (both membrane permeable) and BZ (membrane impermeable) all produced similar outward currents, the implication is that all these agents were acting extracellularly to produce these responses, *via* a common external binding site, unrelated to surface CA activity (Tong *et al*., 2000); the identity of this binding site, however, is currently unknown. It is well known that sulphonamides can interact with many different types of cellular target site to produce a variety of pharmacological effects (Casini *et al*., 2002). Alternatively, BZ may not be as membrane impermeable as generally believed (C. Supuran, University of Florence, Italy; personal communication), and all the three CA inhibitors were interacting with a common intracellular binding sites/receptor to induce outward currents *via* a CA-independent mechanism.

According to Leniger *et al*. (2002), ACTZ can also produce an acidic shift in *intracellular* neuronal pH (although, see Munsch & Pape, 1999). By inference from the work of Church (1992; 1999), this would be expected to *depress* HVA $Ca^{2+}$ conductances, and therefore $Ca^{2+}$-mediated potentials, which is opposite to the enhancing effects on L-type $Ca^{2+}$ tail current and sAHP produced by TPM and ACTZ in our cells. Thus, if these agents were both producing a similar fall in $pH_o$/$pH_i$ *via* CA inhibition, then this could not explain their common action in enhancing the sAHP. Moreover, the latter response to TPM and ACTZ was still observed in a HEPES-buffered medium (despite depression of the control sAHP; Church, 1992), further ruling out the involvement of CA. It is worth noting that ACTZ and BZ occluded the effects of TPM on the outward current response, but were *additive* in producing the enhancement/prolongation effect on the sAHP and s$I_{AHP}$ tail current; this could be taken as evidence for different underlying response mechanisms with different pharmacological sensitivities to these compounds. Although Leniger *et al*. (2002) found no change in neuronal $pH_i$ following BZ application (thus supporting the idea that it is membrane impermeant), as with the outward current response, we are unable to conclude whether the sAHP modulatory effect of TPM and the CA inhibitors is indeed mediated extracellularly or intracellularly (or both).

### *Conclusions and general implications for anticonvulsive therapy*

In conclusion, our study suggests that TPM and possibly other structurally related compounds (e.g. zonisamide) may have common anticonvulsant mechanism of action by inducing neuronal outward currents and enhancing the activity of cortical L-type $Ca^{2+}$ channels, therefore indirectly enhancing the sAHP. Whether other L-type channels, for example, on cardiac, or smooth muscle, are also affected by TPM remains to be tested; however, the different subunit properties of L-channels at these different sites could explain its apparently selective effects on neuronal L-currents. Cardiac, smooth muscle and brain L-channels are known to contain alpha 1C or alpha 1D-type pore-forming subunits (Bell *et al*., 2001), but variation in splice forms and their association with different beta subunits on neurones could confer them with distinct functional and pharmacological properties (Catterall, 1998).

We tested doses of TPM in the range 1–50 $\mu$M (most of our reversible effects were observed at 20 $\mu$M), which is within the therapeutically effective range of free serum levels of TPM observed clinically ($\sim$7–100 $\mu$M; Wolf *et al*., 2000). On the basis of our data, we propose that the sAHP enhancement effect may predominate at low TPM plasma levels, together with the slow hyperpolarization/conductance increase effect, to give the main anticonvulsant action; whether these effects override or act in combination with other proposed effects of TPM on $Na^+$ channels, $GABA_A$ receptors and AMPA/kainate glutamate receptors (Angehagen *et al*., 2003) may be difficult to assess. Interestingly, the relevance of the observed *in vitro* effects of TPM for modulating human motor cortical excitability was recently addressed by Reis *et al*. (2002), who suggested that ion channel-blocking mechanisms may not be so important. Despite the ongoing uncertainties over the contributory mechanisms of action of TPM, the importance of L-$Ca^{2+}$ channels in epileptiform bursting is already well established (Straub *et al*., 2000); therefore, a focus on how TPM modulates cortical L-channel activity, and also how dihydropyridine L-channel blockers, already known to possess anticonvulsant activity (De Sarro *et al*., 1992) might possibly interfere with the anticonvulsive actions of TPM *in vivo* should prove highly informative.

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