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Investigation of the role of intracellular Ca^{2+} stores in generation of the muscarinic agonist-induced slow afterdepolarization (sADP) in guinea-pig olfactory cortical neurones *in vitro*

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1 Intracellular recordings were made from guinea-pig olfactory cortical brain slice neurones to assess the possible role of intracellular Ca^{2+} stores in the generation of the slow post-stimulus afterdepolarization (sADP) and its underlying tail current (I_{ADP}), induced by muscarinic receptor activation.

2 Caffeine or theophylline (0.5–3 mM) reduced the amplitude of the I_{ADP} (measured under 'hybrid' voltage clamp) induced in the presence of the muscarinic agonist oxotremorine-M (OXO-M, 10 μM) by up to 96%, without affecting membrane properties or muscarinic depolarization of these neurones.

3 The L-type Ca^{2+} channel blocker nifedipine (1, 10 μM) also inhibited I_{ADP} (by up to 46%), while ryanodine (10 μM) (a blocker of Ca^{2+} release from internal stores) produced a small ($\sim$10%) reduction in I_{ADP} amplitude; however, neither 10 μM dantrolene (another internal Ca^{2+} release blocker) nor the intracellular Ca^{2+} store re-uptake inhibitors thapsigargin (3 μM) or cyclopiazonic acid (CPA, 15 μM) affected I_{ADP} amplitude.

4 IBMX (100 μM), a phosphodiesterase inhibitor, also had no effect on I_{ADP}. Furthermore, inhibition of I_{ADP} by caffeine was not reversed by co-application of 100 μM adenosine.

5 Caffeine (3 mM) or nifedipine (10 μM) reduced the duration of presumed Ca^{2+} spikes revealed by intracellular Cs^{+} loading. When applied in combination, nifedipine and caffeine effects were occlusive, rather than additive, suggesting a common site of action on L-type calcium channels.

6 We conclude that Ca^{2+}-induced Ca^{2+} release (CICR) from internal stores does not contribute significantly to muscarinic I_{ADP} generation in olfactory cortical neurones. However caffeine and theophylline, which enhance CICR in other systems, blocked I_{ADP} induction. We suggest that this action might involve a combination of L-type voltage-gated Ca^{2+} channel blockade, and a direct inhibitory action on the putative I_{ADP} K^{+} conductance.

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Abbreviations: 1**S**,3**R**-ACPD, 1**S**,3**R**-aminocyclopentane-1,3-dicarboxylic acid; cyclic AMP, 3′:5′-cyclic adenosine monophosphate; CICR, Ca^{2+}-induced Ca^{2+} release; CPA, cyclopiazonic acid; DMSO, dimethylsulphoxide; E.P.S.P, excitatory post-synaptic potential; I_{ADP}, afterdepolarization current; I_{AHP}, afterhyperpolarization current; IBMX, 3-isobutyl 1-methylxanthine; OXO-M, oxotremorine-M; PSP, postsynaptic potential; sADP, slow afterdepolarization; sAHP, slow afterhyperpolarization; ω-CTx GVIA, ω-conotoxin GVIA

Introduction

A wide variety of mammalian central neurones have been shown to exhibit afterdepolarizing responses following stimuli ranging from a single spike (Higashi *et al*., 1993; Haj-Dahmane & Andrade, 1997) to multiple spike discharges (Constanti *et al*., 1993; Libri *et al*., 1996). Such afterpotentials are important in governing neuronal repetitive firing, and can either occur intrinsically (Costa *et al*., 1991; Li & Hatton, 1997), or appear following activation of various receptor systems e.g. muscarinic receptors (Constanti *et al*., 1993; Haj-Dahmane & Andrade, 1998), metabotropic glutamate receptors (Greene *et al*., 1994; Libri *et al*., 1997), 5-hydroxytryptamine (5-HT) receptors (Araneda & Andrade, 1991) or even $GABA_A$-operated channels (Cerne & Spain, 1997). The dependence of some of these spike afterdepolarizations on cellular Ca^{2+} entry has been confirmed by studies on cortical neurones (Schwindt *et al*., 1988; Andrade, 1991), as well as other systems such as dorsal root ganglia (White *et al*., 1989) and supraoptic nucleus neurones (Li *et al*., 1995). Furthermore, it has been reported that L-type voltage-gated Ca^{2+} channels and release of Ca^{2+} from intracellular stores (Ca^{2+}-induced Ca^{2+} release; CICR) play an important role in generation of intrinsic depolarizing afterpotentials in the latter neurone type (Li & Hatton, 1997).

In vitro exposure of adult olfactory cortical neurones to muscarinic (or metabotropic glutamate) agonists has been shown to induce postsynaptic excitatory effects such as a slow depolarization, repetitive spike discharge, and appearance of a slow post-stimulus afterdepolarizing potential (sADP), which was strongly reliant on Ca^{2+} influx, and believed to involve a Ca^{2+}-dependent *de*- and subsequent *re*-activation of a novel potassium conductance (Constanti & Bagetta, 1991; Constanti *et al*., 1993; Libri *et al*., 1994; 1996). This phenomenon was thought to contribute to the characteristically prolonged muscarinic (or metabotropic) excitation of olfactory and perhaps other mammalian cortical neurones, and may there-

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fore be implicated in the formation of memory traces, or the induction and maintenance of epileptiform discharges under certain conditions (Constanti *et al*., 1993; Libri *et al*., 1997). However, the mode of Ca^{2+} entry or the possible involvement of releasable intracellular Ca^{2+} stores in generation of the olfactory cortical sADP have not been investigated.

Methylxanthines such as caffeine and theophylline have a wide range of pharmacological actions on central neurones (e.g. direct interaction with voltage-gated Ca^{2+} channels, inhibition of phosphodiesterases and direct antagonism of adenosine receptors; for review see Fredholm *et al*., 1999). However, one of the most well known actions of these compounds is the mobilization of Ca^{2+} from neuronal intracellular stores through interaction with intracellular ryanodine receptors (for reviews see Nehlig *et al*., 1992; Sawynok & Yaksh, 1993; Kuba, 1994). In view of the findings of Li & Hatton (1997) showing that caffeine-induced Ca^{2+} release from internal stores could enhance the amplitude of Ca^{2+}-dependent depolarizing afterpotentials recorded in supraoptic nucleus neurones, and also taking into account previous reports of the role of neuronal Ca^{2+} entry and intracellular Ca^{2+} release in the generation of the slow afterhyperpolarization (sAHP) in guinea-pig vagal neurones (Sah & McLachlan, 1991), rabbit otic ganglion cells (Yoshizaki *et al*., 1995) or hippocampal pyramidal cells (Tanabe *et al*., 1998), we were interested in examining the possible role of Ca^{2+} release from caffeine-sensitive intracellular stores in olfactory cortical sADP generation. The effects of caffeine, and some other agents which modulate calcium entry/release from intracellular stores, upon muscarinic induction of the sADP and its underlying inward tail current (I_{ADP}, measured under 'hybrid' voltage clamp) were therefore investigated under the present conditions. A preliminary report of these findings has recently been presented to the British Pharmacological Society (Postlethwaite *et al*., 1999).

Methods

Preparation and storage of brain slices

Slices of guinea-pig olfactory cortex were prepared and stored as described previously by Constanti *et al*. (1993). Briefly, adult albino guinea-pigs (250–400 g, of either sex) were anaesthetized using halothane, decapitated, and the brain rapidly removed. A rectangular block of tissue was then prepared incorporating the lateral olfactory tract, the olfactory cortex and surrounding regions, which was stuck onto the cutting stage of a Campden vibroslice/M tissue cutter with cyanoacrylate glue. Transverse slices of ~450 μm thickness were then cut in ice cold (~4°C Krebs solution), transferred to a storage chamber and incubated in oxygenated Krebs at 32°C for 30 min–1 h before transferring to the recording chamber maintained at 30°C. The composition of the Kreb's solution was (mM): NaCl 118; KCl 3; $CaCl_2$ 1.5; $NaHCO_3$ 25; $MgCl_2.6H_2O$ 1 and D-glucose 11 (bubbled with 95% O_2 : 5% CO_2, pH 7.4).

Recording and stimulation

Recordings were taken from neurones in cell layers II–III of the periamygdaloid area of the slices with glass microelectrodes filled with 4 M potassium acetate (tip resistance 50–80 MΩ), using conventional intracellular current-clamp/voltage-clamp techniques. An Axoclamp 2A sample-and-hold preamplifier was used with switching frequency 2–3 kHz, and 30% duty cycle. Membrane resistance and firing properties were measured by passing positive or negative current pulses of increasing intensities (~0.5–3 nA, 160 ms duration) and the resulting electrotonic potential recorded. During applications of muscarinic or metabotropic receptor agonists, the membrane potential was routinely depolarized to −70 mV (close to firing threshold) by applying steady depolarizing current; tests with other compounds, or experiments to test the effects of caffeine on the muscarinic-induced depolarization, were carried out at resting potential (~−84 mV).

For evoked synaptic responses, a bipolar nichrome wire electrode (50 μM diameter, insulated except at the tip) was used to deliver orthodromic stimuli to association fibres in cortical layer III projecting to layer II–III neurones. Postsynaptic potentials (PSPs) were evoked using stimuli of increasing intensity (5–30 V) and constant duration (200 μs) delivered by a Digitimer isolated DS2 stimulator. Recordings of synaptic potentials were routinely taken at −84 mV membrane potential for comparison, and stimulus strength was adjusted so that the evoked excitatory PSP was just subthreshold for evoking an orthodromic action potential. All recordings of membrane and synaptic responses were taken before and during application of drugs so that each neurone served as its own control.

Membrane current and voltage signals were sampled and then fed to a computer (Viglen 4DX2-66, Viglen Ltd., U.K.) *via* a Digidata 1200 analogue-to-digital interface (Axon instruments, Foster City, CA, U.S.A.) using pCLAMP 6.03 software (Axon instruments) for recording and subsequent off-line analysis. Data were also recorded on a Gould RS3200 ink-jet chart recorder. Data are presented as mean ± s.e.mean, and where appropriate, differences between groups are expressed as percentage change in comparison to control. Statistical significance between data groups was assessed by paired *t*-tests performed on the original recorded data, prior to any transformation or calculation of percentages.

Drugs

Drugs used in this study included caffeine, theophylline and nifedipine (all from Sigma Ltd, U.K.), 3-isobutyl-1-methylxanthine (IBMX), ryanodine, dantrolene, thapsigargin, cyclopiazonic acid (CPA) (all kindly donated by Sigma Ltd, U.K.), oxotremorine-M iodide (OXO-M) (Semat Ltd, U.K.), 1**S**,3**R**-aminocyclopentane-1,3-dicarboxylic acid (1**S**,3**R**-ACPD) (Tocris Cookson Ltd, Bristol, U.K.) and ω-conotoxin GVIA (ω-CTx GVIA) (Alomone Laboratories, Jerusalem, Israel). Except where specified, all drugs were prepared in Krebs solution from stock solutions predissolved in water. Caffeine and theophylline were routinely dissolved directly into Krebs solution on the day of the experiment, whereas IBMX, thapsigargin, dantrolene and nifedipine were pre-dissolved in dimethylsulphoxide (DMSO); 1**S**,3**R**-ACPD was pre-dissolved in 1 : 1 equivalent of 100 mM sodium hydroxide (NaOH) solution. ω-CTx GVIA was dissolved in distilled water at 100 μM; small aliquots were then prepared and stored at −20°C until required. The aliquots were diluted to a final concentration of 200 nM in bathing medium immediately before use. To minimize nonspecific binding of toxin to plastic tubes, all toxin solutions contained 0.1 mg/ml cytochrome C and 1 mg/ml lysozyme (both from Sigma Ltd., U.K.). (Magistretti *et al*., 1999). Nifedipine, dantrolene and cytochrome C were weighed and dissolved in semi-darkness, due to their light sensitive nature, and subsequently stored in containers wrapped in silver foil to exclude light. Precautions were also taken to minimise their exposure to light during

applications of these agents to cells. Final bath concentrations of DMSO (up to 0.5%), NaOH (up to 0.1%), cytochrome C (0.1 mg/ml) or lysozyme (1 mg/ml) had no deleterious effects on neuronal membrane properties or muscarinic/metabotropic responsiveness.

Results

Intrinsic neuronal membrane properties

Stable intracellular recordings of 1–5 h duration were obtained from a total of 63 guinea-pig olfactory cortical neurones. In control solution, neurones electrophysiologically identified as 'deep' (layer II–III) cells (Libri *et al*., 1994) had a mean resting membrane potential of -84.3 ± 0.3 mV, spike amplitude (measured from baseline) of 114 ± 0.8 mV, and a mean resting input resistance (calculated from <20 mV hyperpolarizing electrotonic potentials) of 40.0 ± 1.5 MΩ; their electrophysiological properties and firing behaviour were typical for pyramidal cells in rodent cerebral cortex (Connors & Gutnick, 1990). Neurones electrophysiologically identified as superficial pyramidal cells ($n=7$) were previously reported to be unresponsive to muscarinic or metabotropic agonists (Libri *et al*., 1994), so were regularly discarded.

Effects of caffeine on muscarinic or metabotropic glutamate receptor responses

A prolonged application (2–5 min) of the muscarinic receptor agonist OXO-M (10 μM) or the metabotropic glutamate agonist 1**S**,3**R**-ACPD (10 μM) to the olfactory neurones resulted in a slow membrane depolarization (mean = 8.3 ± 0.4 mV in OXO-M, $n=36$; or 5.0 ± 1.5 mV in 1**S**,3**R**-ACPD, $n=3$) with superimposed repetitive spike discharge (~10 Hz), coupled with an increase in membrane input resistance (8.5 ± 3.6% in OXO-M or 22.4 ± 7.6% in 1**S**,3**R**-ACPD, measured from −70 mV membrane potential). Exposure of these responding neurones to 3 mM caffeine (15 min) did not notably alter the slow depolarization response profile and subsequent repetitive firing induced at −70 mV membrane potential by either OXO-M ($n=3$) or 1**S**,3**R**-ACPD ($n=3$; not shown). Caffeine (3 mM) also had no significant effect on the muscarinic depolarization amplitude measured from a more negative membrane potential (−84 mV), where no induced firing occurred (mean amplitude = 13 ± 4 mV in OXO-M, and 12.5 ± 0.5 in OXO-M plus 3 mM caffeine; $P>0.05$; $n=3$; Figure 1A). 3 mM caffeine, applied alone in control solution ($n=5$), also had no measurable effect on the intrinsic membrane properties of recorded cells.

Effects of caffeine on sADP and I_{ADP}

As previously reported (Constanti *et al*., 1993; Libri *et al*., 1997), application of 10 μM OXO-M or 10 μM 1**S**,3**R**-ACPD induced the appearance of a sADP in response to a large (1.6 s) depolarizing stimulus, which replaced the sAHP evoked in control solution (Figure 1B). The mean sADP amplitude (measured at −70 mV) was 8.7 ± 0.4 mV (range 4–12 mV) in OXO-M ($n=38$) and 7.0 ± 1.5 mV (range 4–9 mV) in the presence of 1**S**,3**R**-ACPD ($n=3$). The underlying inward tail current (I_{ADP}) revealed under 'hybrid' voltage clamp (Penne-

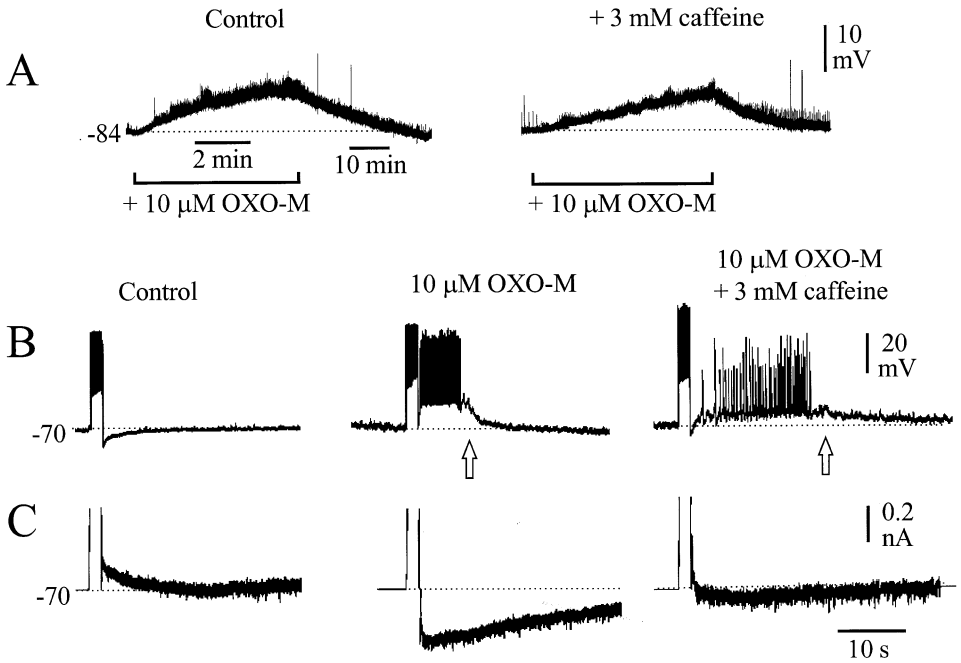

Figure 1 Effects of 3 mM caffeine on the muscarinic depolarization, sADP and I_{ADP} observed in olfactory cortical neurones. (A) Shows the effect of applying 10 μM oxotremorine-M (OXO-M, 6 min; indicated by solid bar below trace) to a neurone at the resting membrane potential (−84 mV) in control solution, and in the presence of 3 mM caffeine. Note that caffeine had no effect on the amplitude of the slow muscarinic depolarization of the cell (continuous chart recording; chart speed was slowed during drug washout). (B) (Different neurone maintained at −70 mV membrane potential). A prolonged depolarizing stimulus (1.6 s, +2 nA) induces a post-stimulus sAHP in control solution (left panel). In the presence of 10 μM OXO-M, the same current injection induces a post-stimulus sADP with superimposed repetitive firing (middle panel). In 3 mM caffeine (15 min) the sADP and associated repetitive firing induced by OXO-M were dramatically reduced (right panel); (in both cases, a small direct negative current was applied at the open arrow leading to a gradual hyperpolarization of the cell to the original 'holding' potential. (C) Corresponding slow tail currents measured under 'hybrid' voltage-clamp at −70 mV. Left panel shows the outward tail current underlying the sAHP recorded in control solution. Middle panel shows the slow inward tail current underlying the sADP (I_{ADP}) recorded in OXO-M. Further addition of caffeine produced a clear reduction in the I_{ADP} amplitude (right panel).

father *et al*., 1985), at −70 mV holding potential, had a mean amplitude of 0.31±0.02 nA (range 0.10–0.67 nA) and mean duration 37.9±1.6 s (range 20–67 s) in OXO-M ($n=42$) (Figure 1C) and 0.28±0.01 nA amplitude, 27.3±3.2 s duration in 1**S**,3**R**-ACPD ($n=3$, not shown). Induction of the sADP (and I_{ADP}) were readily reversible upon washout of either OXO-M or 1**S**,3**R**-ACPD from the bathing solution.

Despite its lack of effect on muscarinic agonist responses, application of caffeine consistently resulted in a dramatic reduction in the amplitude of the OXO-M-induced sADP and its underlying tail current I_{ADP} (Figure 1B,C). This reduction was concentration-dependent, being reduced by 31.6±1.4% and 30.2±3.0% in 0.5 mM ($n=3$), 26.7±14.5% and 51.5±15.5% in 1 mM ($n=3$), 60.8±5.8% and 74.5±9.1% in 2 mM ($n=3$) and 64.1±15.0% and 74.2±8.1% in 3 mM caffeine respectively ($n=8$) (all reductions significantly different from control, by paired *t*-tests performed on original untransformed data; $P<0.05$) (Figure 2A). Caffeine (3 mM) also significantly reduced the sADP and I_{ADP} induced by 10 μM 1**S**,3**R**-ACPD (77.8±13.8% and 73.3±15.0% respectively; $n=3$, $P<0.05$, by *t*-tests: not shown). All effects were readily reversible after 15–20 min of caffeine washout.

Theophylline, another methylxanthine which can release Ca^{2+} from intracellular stores (Smith *et al*., 1983), also significantly inhibited I_{ADP} in a concentration-dependent reversible manner, with a similar potency to caffeine; mean reduction was 41.1±8.4% in 0.5 mM, 68.9±9.3% in 1 mM, 85.5±0.4% in 2 mM and 96.2±3.9% in 3 mM theophylline ($P<0.05$, $n=3$; *t*-tests; Figure 2B).

Effects of caffeine on evoked synaptic potentials

Synaptic potentials could be elicited using an external stimulating electrode placed in layer III of the cortical slice. Focal stimulation (10–30 V; 0.2 ms) of the local association fibres resulted in the appearance of the characteristic excitatory/inhibitory postsynaptic potential (e.p.s.p/i.p.s.p) complex as described previously (Libri *et al*., 1996; 1997). No repetitive spike discharges were superimposed on the e.p.s.p, even following stimuli of maximal intensity (30 V) or depolarizing the membrane potential up to −60 mV ($n=3$); the threshold for appearance of an action potential was around −65 mV. The mean amplitude of evoked e.p.s.ps in control solution at −84 mV membrane potential was 14.6±0.7 mV ($n=28$). In the presence of 10 μM OXO-M, the mean e.p.s.p amplitude was reduced by 58±3% ($n=28$), and in 10 μM 1**S**,3**R**-ACPD it was reduced by 80±10% ($n=3$) (both reductions significant; $P<0.05$, *t*-tests; *c.f*. Libri *et al*., 1997). By contrast, in 3 mM caffeine, there was a clear and consistent augmentation of evoked synaptic transmission, the peak e.p.s.p amplitude being enhanced by 93±33% ($n=5$, $P<0.05$, *t*-test) (most probably due to inhibition of endogenous adenosine activity on presynaptic adenosine receptors; Motley & Collins, 1983; Haas & Greene, 1988) (Figure 3).

Blockade of Ca^{2+} entry suppresses I_{ADP}

Blockade of L-type voltage-gated Ca^{2+} channels has been shown to inhibit the sAHP in hippocampal CA3 cells (Tanabe *et al*., 1998); the possible contribution of these channels towards sADP generation in olfactory neurones was therefore investigated by application of the selective L-type calcium channel blocker nifedipine (1 and 10 μM; Bean, 1989; Kostyuk, 1999). The amplitude of the OXO-M-induced I_{ADP} was

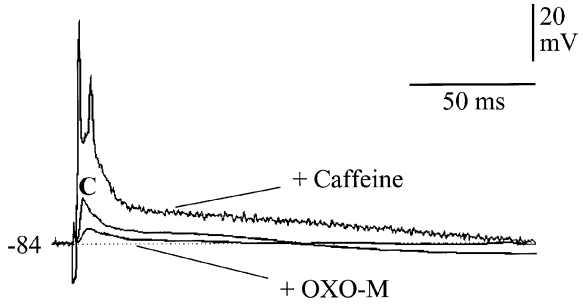

Figure 3 Postsynaptic potentials (p.s.ps) recorded intracellularly at −84 mV membrane potential in response to orthodromic electrical stimuli (25 V, 0.2 ms) delivered locally to association fibre terminals; the stimulus strength was just subthreshold for evoking action potentials in control solution. Superimposed traces show p.s.ps evoked during the control period (C), after a 2 min bath-application of 10 μM oxotremorine-M (OXO-M) or after 15 min in 3 mM caffeine. A 30 min washout period was allowed between each drug application. Note the reduction in p.s.p amplitude induced by OXO-M, and the marked enhancement of synaptic transmission in the presence of caffeine (p.s.p now exceeding spike threshold).

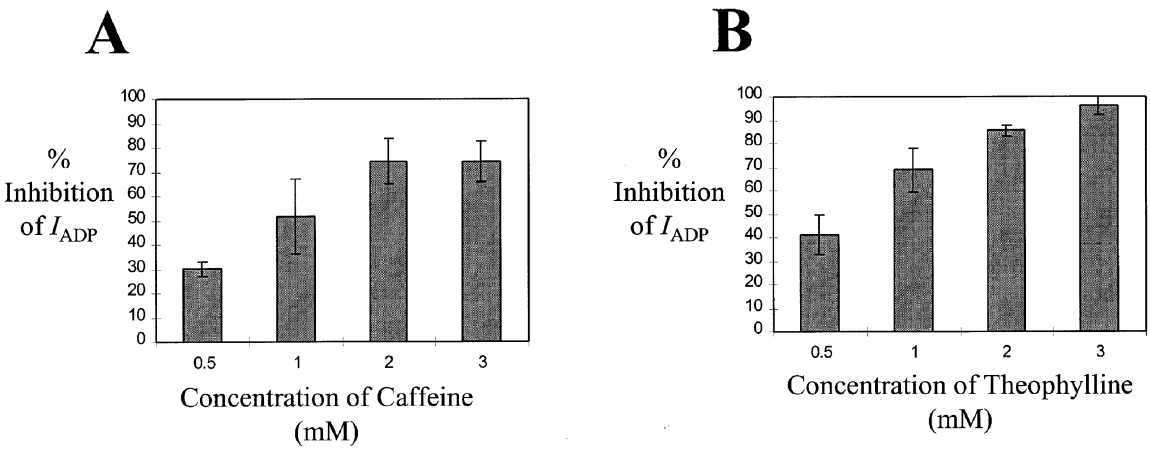

Figure 2 The inhibition of the I_{ADP} by caffeine or theophylline is concentration-dependent. (A) shows the percentage reduction in I_{ADP} amplitude in the presence of 0.5 mM, 1 mM, 2 mM ($n=3$ at each concentration) or 3 mM ($n=8$) caffeine respectively. (B) shows the similar reduction of I_{ADP} amplitude in 0.5 mM, 1 mM, 2 mM or 3 mM theophylline ($n=3$ at each concentration). Data are presented as mean percentage reduction of the peak I_{ADP} amplitude (±s.e.mean) induced by caffeine or theophylline in the presence of 10 μM OXO-M. All measurements were performed at a holding membrane potential of −70 mV, following a 'hybrid' voltage clamp protocol; a set current stimulus of +2 nA, 1.6 s was used throughout.

reduced in a concentration-dependent fashion by application of nifedipine at 1 μM or 10 μM concentration. In 1 μM nifedipine, the mean I_{ADP} amplitude was reduced from 0.10 ± 0.1 nA to 0.08 ± 0.01 nA, a reduction of $28.5 \pm 1.5\%$ ($n=3$) while at 10 μM nifedipine, the mean I_{ADP} amplitude was reduced from 0.39 ± 0.09 nA to 0.21 ± 0.05 nA, a decrease of $41.7 \pm 4.8\%$ ($n=4$) (both reductions significantly different from control; $P<0.05$; Figure 4); the effects of nifedipine were reversed after 40 min of washout.

By contrast, the peptide toxin ω-conotoxin GVIA (ω-CTx-GVIA) (200 nM, $n=4$), which is known to selectively block N-type Ca^{2+} channels in central neurones (Magistretti *et al*., 1999), produced only a small and variable reduction in the OXO-M-induced I_{ADP} (amplitude decreased from 0.16 ± 0.04 nA to 0.13 ± 0.05 nA, a mean reduction of 18.8%); this effect was not significantly different from control ($P>0.05$). Note that this concentration of ω-CTx-GVIA was previously found to reduce the amplitudes of Ca^{2+}-dependent spike afterdepolarizations in supraoptic nucleus neurones (Li & Hatton, 1997).

Effects of ryanodine receptor blockers and Ca^{2+} store uptake inhibitors on I_{ADP}

The possible role of CICR in generating the olfactory cortical sADP and the underlying I_{ADP} was investigated using the membrane-permeable inhibitors of intracellular Ca^{2+} release, ryanodine and dantrolene (Henzi & MacDermott, 1992). Further to this, the effects of intracellular Ca^{2+} store depletion were also tested by using the membrane permeable Ca^{2+} store uptake inhibitors thapsigargin and cyclopiazonic acid (CPA; Markram *et al*., 1995). Bath-application of ryanodine (10 μM, 15 min) reduced the I_{ADP} amplitude from 0.38 ± 0.06 nA to 0.35 ± 0.06 nA, a small but significant amount (mean reduction $= 9.9 \pm 4.2\%$, $P<0.05$, $n=7$, *t*-test; Figure 5A), while application of dantrolene (10 μM, $n=3$) for up to 40 min failed to reduce the sADP or I_{ADP} amplitudes (Table 1; Figure 5B).

Thapsigargin and CPA have previously been shown to suppress neuronal Ca^{2+} uptake into intracellular stores, by inhibiting the Ca^{2+}. ATPase in the endoplasmic reticulim, making less Ca^{2+} available for subsequent release (Markram *et al*., 1995). However, neither thapsigargin (3 μM, $n=7$) nor CPA (15 μM, $n=3$) reduced the amplitude of I_{ADP}, even after 30–40 min exposure to these agents (Table 1; Figure 5C,D).

Furthermore, in an attempt to determine whether the observed blocking actions of caffeine or ryanodine on the I_{ADP} could be mediated by Ca^{2+} release from intracellular stores, we examined the effects of these compounds in the presence of 3 μM thapsigargin; however, prior exposure (15 min) of neurones to this agent had no significant effect on the I_{ADP}-blocking actions of either 3 mM caffeine ($n=3$) or 10 μM ryanodine ($n=3$) ($P>0.05$ in both cases; *t*-tests).

Other possible actions of caffeine

Caffeine is known to have several different sites of action (Fredholm *et al*., 1999) apart from its classical interaction with intracellular Ca^{2+} store release channels (Sawynok & Yaksh, 1993; Kuba, 1994). Thus, caffeine also inhibits the enzyme

Table 1 The effects of intracellular Ca^{2+} release modulators upon peak I_{ADP} amplitude recorded in olfactory cortical neurones

	I_{ADP} *amplitude* (nA)	
Drug	*Control*	*+ Treatment*
Ryanodine (10 μM)	0.38 ± 0.06 ($n=7$)	0.35 ± 0.06* ($n=7$)
Dantrolene (10 μM)	0.28 ± 0.04 ($n=3$)	0.25 ± 0.03 ($n=3$)
Thapsigargin (3 μM)	0.47 ± 0.13 ($n=7$)	0.43 ± 0.12 ($n=7$)
Cyclopiazonic acid (15 μM)	$0.26 = 0.06$ ($n=3$)	0.23 ± 0.06 ($n=3$)

Ryanodine and dantrolene interfere with release of Ca^{2+} from intracellular stores, whereas thapsigargin and cyclopiazonic acid (CPA) prevent Ca^{2+} reuptake into the stores, depleting them of Ca^{2+}. All recordings of peak I_{ADP} amplitude were taken under 'hybrid' voltage clamp at a holding potential of -70 mV in the presence of 10 μM OXO-M. In all cases (apart from ryanodine), there was no detectable difference between the mean I_{ADP} amplitudes measured before or after administration of drug. *The reduction of I_{ADP} amplitude by ryanodine was significant ($P<0.05$, *t*-test). Values in parentheses refer to the numbers of neurones used for each investigation.

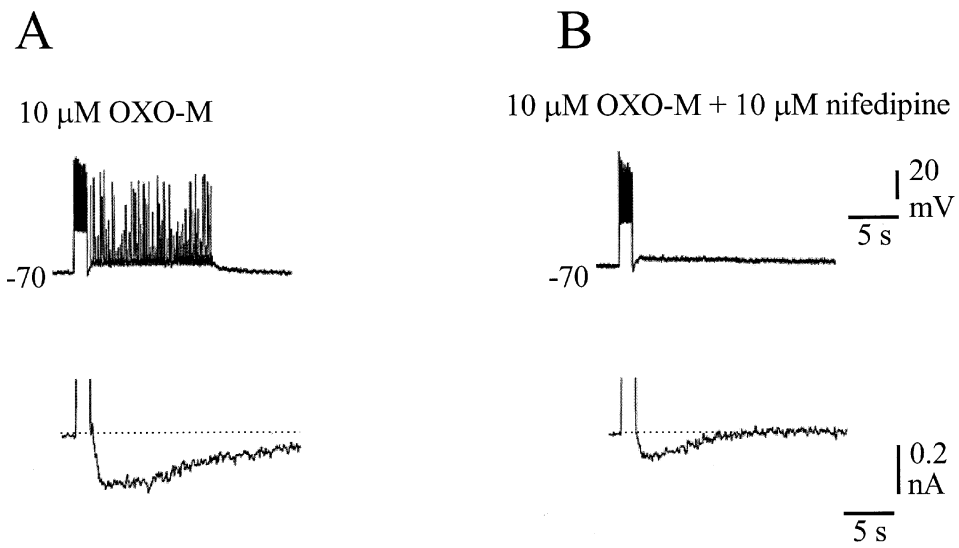

Figure 4 Inhibitory effects of the L-type Ca^{2+} channel blocker nifedipine on the sADP and I_{ADP} measured in a single olfactory cortical neurone. (A) shows the control sADP and I_{ADP} recorded in 10 μM OXO-M at -70 mV membrane holding potential respectively. (B) shows the sADP and I_{ADP} recorded in the same neurone in the presence of 10 μM OXO-M and 10 μM nifedipine; note the reduction in sADP and I_{ADP} amplitude. Stimulus parameters were +2 nA, 1.6 s throughout.

phosphodiesterase, leading to an increase in intracellular cyclic AMP concentration (Sawynok & Yaksh, 1993), as well as being a direct antagonist at adenosine receptors (Daly *et al*., 1981; Greene *et al*., 1985). To test the former possibility, some experiments were carried out in the presence of the broad-spectrum phosphodiesterase inhibitor IBMX. Out of three neurones tested with 100 mM IBMX (in the continued presence of 10 μM OXO-M), none showed any reduction of the sADP or I_{ADP} amplitude compared to OXO-M alone, even after 30 min IBMX exposure; this suggested that phosphodiesterase inhibition could not account for the block of the I_{ADP} by caffeine.

In light of the previously reported inhibition of the hippocampal sAHP (and its underlying I_{AHP}) by caffeine (Haas & Greene, 1988), and its enhancement by applied adenosine (Haas & Greene, 1984), the possibility that caffeine might be affecting I_{ADP} by behaving as an antagonist of endogenously-released adenosine (possibly important for I_{ADP} generation) was also investigated (*c.f.* Motley & Collins, 1983). In these experiments, the I_{ADP} was initially recorded in the presence of 10 μM OXO-M alone, and then in the presence of 3 mM caffeine, which produced an almost complete blockade of the tail current (mean reduction = 86.8 ± 5.6%, $n = 3$) and enhanced the evoked synaptic potentials (mean enhancement = 144.2 ± 66.7%, relative to amplitude in OXO-M; $n = 3$) (Figure 6). Following this, 100 μM adenosine was added in an attempt to counteract any possible antagonism of endogenous adenosine receptors produced by caffeine. After 15 min exposure, the e.p.s.p amplitude was significantly reduced from 18.1 ± 1.0 mV to 5.9 ± 2.1 mV (mean reduction = 73.1 ± 14.7% relative to the enhanced level in caffeine/OXO-M; $n = 3$; $P < 0.05$, *t*-test), suggesting that adenosine was reversing the effect of caffeine presynaptically. However, there was no detectable difference between the I_{ADP} amplitude recorded in caffeine, or with added adenosine, suggesting that caffeine blockade of adenosine receptors was not responsible for the observed reduction in the I_{ADP} (Figure 6D).

A possible direct blockade of Ca^{2+} channels by caffeine

There are several reports showing a direct interaction of caffeine with calcium currents in cell membranes of muscle cells (Zholos *et al*., 1991; Varro *et al*., 1993; Yoshino *et al*., 1996), indicating an overall decrease in conductance through these channels. To examine whether caffeine was inhibiting I_{ADP} by blocking voltage-activated Ca^{2+} currents in the olfactory cortical neurones, cells were recorded with 2 M caesium chloride-filled electrodes. It has previously been shown that intracellular Cs^+ loading blocks voltage-gated K^+ channels, thereby delaying repolarization after an action potential, and revealing a characteristic plateau potential resulting from activation of voltage-gated Ca^{2+} channels (Galvan *et al*., 1985; Libri *et al*., 1996). Cells impaled with caesium chloride electrodes were routinely left for at least 15 min to allow the Cs^+ to diffuse into the cell interior, after which the evoked action potential duration was lengthened accordingly (Figure 7A,B, left panels). The spike half-width, measured at half peak amplitude from baseline, was taken as a standard measure during the Cs^+-loading experiments, and care was taken to ensure that all recordings were made at the same membrane potential. Superfusion of the neurones with 1 or 10 μM nifedipine significantly reduced the half-width of the first action potential in a concentration-dependent manner: at 1 μM, the mean half-width was reduced from 65.7 ± 9.1 ms to 50.1 ± 10.5 ms (mean reduction 27.3 ± 7.1%; $P < 0.05$, $n = 5$; not shown), whereas at 10 μM, the half-width was reduced from 55.5 ± 8.0 ms to 35.4 ± 8.5 ms (mean reduction 42.8 ± 8.8%, $n = 8$; $P < 0.05$, *t*-test), indicating that the plateau

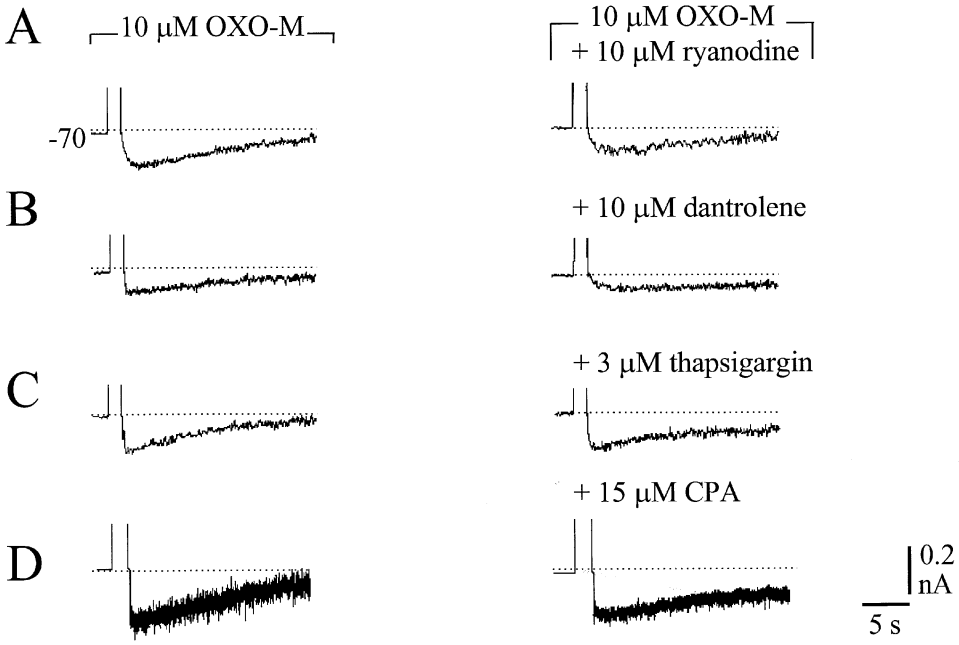

Figure 5 Effects of intracellular Ca^{2+} release modulators on I_{ADP} amplitude in the presence of 10 μM OXO-M. Left panels of each section show the control I_{ADP} evoked in OXO-M and right panels show I_{ADP} in the presence of drug. (A) Superfusion of 10 μM ryanodine caused a small but significant depression of the I_{ADP} (~10%), whereas 10 μM dantrolene (B), 3 μM thapsigargin (C) or 15 μM cyclopiazonic acid (CPA) (D) had no obvious effect. In each experiment, I_{ADP} was recorded under 'hybrid' voltage clamp at −70 mV holding potential, and was elicited using set stimulus parameters (+2 nA, 1.6 s). Scale bars refers to all recordings; (A–D were obtained from different neurones).

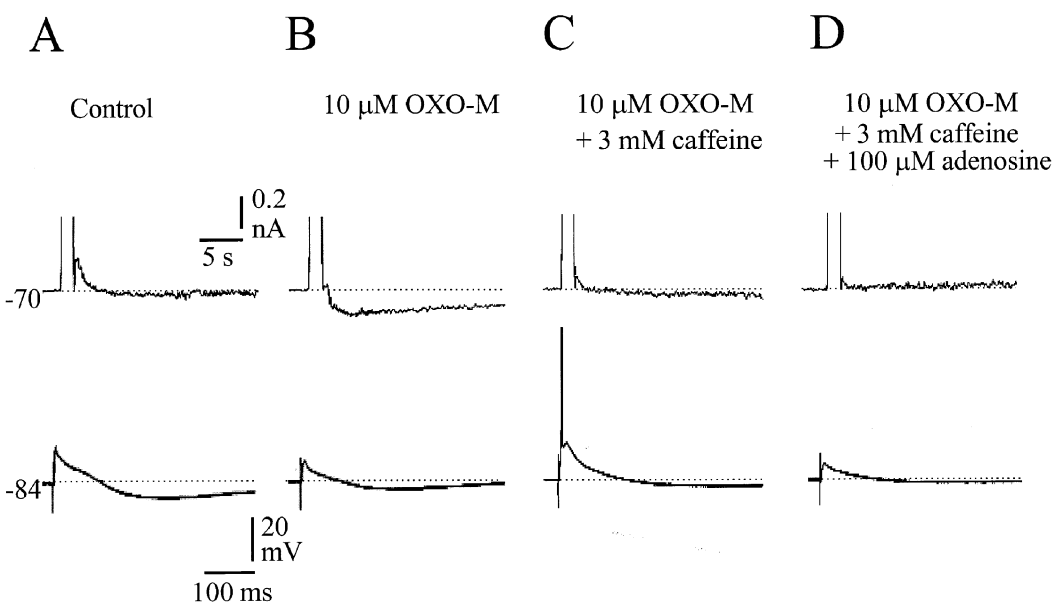

Figure 6 Attempts to reserve the effects of caffeine with applied adenosine. (A) shows the I_{ADP} tail current (top) and the evoked synaptic potential (bottom) recorded in control solution. (B) I_{ADP} and evoked synaptic potential recorded in the presence of 10 μM OXO-M alone. (C) Inhibition of the I_{ADP} and enhancement of the e.p.s.p. produced by adding 3 mM caffeine. (D) Application of 100 μM adenosine failed to reverse the inhibition of the I_{ADP} by caffeine, although the e.p.s.p. was reduced. Top scale bars refer to all current traces and bottom scale bars refer to all voltage traces. I_{AHP} and I_{ADP} were elicited under 'hybrid' voltage clamp at -70 mV holding potential using set stimulus parameters ($+2$ nA, 1.6 s). Synaptic potentials were all recorded at -84 mV membrane potential, using identical stimulus parameters throughout (2.0 V, 0.8 ms).

resulted, in part, from the activation of L-type voltage-gated Ca^{2+} channels (Figure 7A, middle panel). Likewise, superfusion of cells with 3 mM caffeine resulted in a significant reduction of the first spike half-width from 57.3 ± 4.4 ms to 33.0 ± 10.8 ms (45.0 ± 15.0% reduction, $n=3$; $P<0.05$, *t*-test; Figure 7B, middle panel). No further reduction of the spike half-width was observed on applying 1 μM nifedipine in the presence of 3 mM caffeine ($n=4$; $P>0.05$; *t*-test; Figure 7A, right panel). Also, in the presence of 10 μM nifedipine, addition of 3 mM caffeine exerted no further significant reduction in spike half-width ($n=3$; $P>0.05$; *t*-test; Figure 7B, right panel). These data strongly suggest that caffeine was predominantly blocking voltage-gated (possibly L-type) Ca^{2+} conductances in the olfactory cortical neurones.

Discussion

The sADP and I_{ADP} *in responding neurones*

The sADP and its underlying tail current I_{ADP} could be readily revealed by application of a muscarinic or metabotropic (glutamate) agonist in neurones from layer II–III of the guinea-pig olfactory cortex as previously reported (Constanti & Bagetta, 1991; Constanti *et al*., 1993; Libri *et al*., 1997). The mechanism of induction of the sADP was originally proposed to involve a Ca^{2+}-dependent inactivation of a novel ongoing K^+ conductance (during the prolonged spike burst), with a subsequent slow reactivation of this current over several seconds, to give the sADP its characteristic long-lasting deflection (Constanti & Bagetta, 1991; Constanti *et al*., 1993). The principal aim of the present series of experiments was to establish whether CICR from intracellular Ca^{2+} stores contributes to this sADP induction process. To this end, a series of compounds were tested, which either augment or inhibit Ca^{2+} release from intracellular stores in other systems.

Caffeine inhibition of the sADP and I_{ADP}

Caffeine is now widely acknowledged as an agent which can enhance CICR from ryanodine-sensitive stores (for review see Fredholm *et al*., 1999), leading to profound effects on the activity of neurones in which this process is essential for normal function. Early studies on intracellular Ca^{2+} release used a relatively high dose of caffeine as standard (10 mM; Marrion & Adams, 1992; Nohmi *et al*., 1992); however, in several experiments where this concentration was applied to olfactory neurones, it led to rapid depolarization and cell death. For this reason, the concentrations of caffeine used in the present work were lowered, although at these doses, it became less likely that this agent was directly interfering with intracellular Ca^{2+} release.

3 mM caffeine was consistently found to inhibit I_{ADP} (and the concomitant sADP) induced by either OXO-M or 1**S**,3**R**-ACPD in a reversible manner; thus caffeine was not acting directly on either receptor system to cause the inhibition, an observation which was further confirmed by the apparent lack of inhibition of the muscarinic (or metabotropic) agonist-induced depolarizations by caffeine during these recordings. It may be noted however, that caffeine has been previously found to directly antagonize the effects of carbachol and the binding of ^{3}H-N-methylscopolamine in rat pancreatic acini (Grosfils *et al*., 1996). Also, the inhibition of the OXO-M-induced I_{ADP} was concentration-dependent over the range of caffeine doses used (0.5–3 mM). The inhibition of I_{ADP} by caffeine was mimicked by theophylline, another member of the methylxanthine group; this inhibition was similar to that of caffeine, and was dose-dependent over a similar concentration range. Thus, the block of I_{ADP} by caffeine was not specific to this methylxanthine. Considering that both caffeine and theophylline can augment release of Ca^{2+} from intracellular stores (Endo, 1977; Smith *et al*., 1983; Kuba, 1994), and taking into account the proposed mechanism of induction of I_{ADP} (i.e.

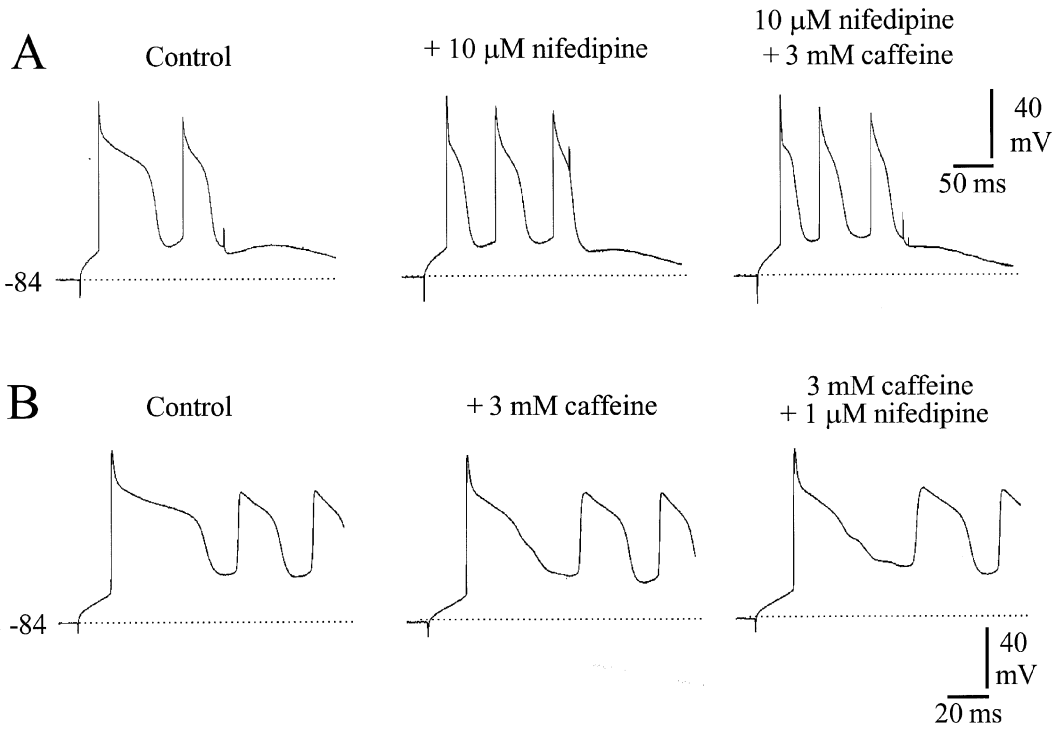

Figure 7 Prolonged action potentials recorded with caesium chloride-filled microelectrodes, revealing calcium plateaus. (A) Depolarizing electrotonic potential evoked in control solution (left panel), with a clear calcium plateau present on the first and subsequent action potentials. In the presence of 10 μM nifedipine (15 min) a clear reduction is observed in the half-width of the initial spike (middle panel); however, no further change in first spike half-width occurred on co-applying nifedipine with 3 mM caffeine (right panel). (B) Recordings made from a different neurone show a similar reduction in initial spike half-width in 3 mM caffeine that was unaffected by further adding 1 μM nifedipine. Scale bars alongside refer to all traces. Current stimulus parameters were identical for each trace (+0.5 nA, 160 ms).

raised intracellular Ca^{2+} leading to a decrease in K^+ conductance; Constanti *et al*., 1993), it seems very unlikely that these agents were further raising the intracellular Ca^{2+} level during a long depolarizing stimulus, since this would be expected to increase the I_{ADP}, amplitude, as seen in the case of the Ca^{2+}-dependent depolarizing afterpotential recorded in supraoptic nucleus neurones (Li & Hatton, 1997). In addition, depletion of putative intracellular Ca^{2+} stores in the olfactory neurones by prior application of thapsigargin had no effect on the blocking action of caffeine on I_{ADP}, thereby suggesting that intracellular Ca^{2+} stores play little or no role in this inhibitory process. Caffeine and theophylline were therefore acting on other systems to cause I_{ADP} inhibition. Even if the olfactory cortical I_{ADP} had some other underlying ionic mechanism such as an increase in nonspecific cationic conductance, as proposed by Haj-Dahmane & Andrade (1998) in prefrontal cortex neurones, then one would still expect an increase in I_{ADP} amplitude in caffeine, since this cortical afterdepolarization was also dependent on intracellular Ca^{2+} concentration.

Intracellular Ca^{2+} stores – a role in I_{ADP} generation?

The importance of Ca^{2+} influx in the generation of the sADP and I_{ADP} allowed speculation about the possible functional role of CICR from intracellular stores in this process. Thus, application of the Ca^{2+} release channel blocker ryanodine caused a small (~10%), yet significant, decrease in amplitude of I_{ADP}, implying that Ca^{2+} stores present within the olfactory neurones, were contributing towards generation of the slow afterpotential response. If so, then these putative stores would appear to have only a very limited role in this type of cellular activity, unlike the generation of depolarizing afterpotentials in the supraoptic nucleus (Li & Hatton, 1997) or the sAHP in hippocampal neurones (Torres *et al*., 1996; Tanabe *et al*., 1998). Furthermore, there was no significant reduction of I_{ADP} amplitude in the presence of the CICR blocker dantrolene, or the intracellular Ca^{2+} store depletors thapsigargin or CPA, again suggesting a minimal involvement of these stores in cortical I_{ADP} generation (*c.f.* supraoptic neurones or hippocampal neurones where these agents clearly reduced afterpotential amplitudes; Li & Hatton, 1997; Torres *et al*., 1996; Tanabe *et al*., 1998). The small blocking effect of ryanodine on I_{ADP} also persisted in the continued presence of thapsigargin, suggesting that intracellular Ca^{2+} release was not involved.

One possibility was that ryanodine reduced I_{ADP} by directly blocking Ca^{2+} currents in the plasma membrane, thereby preventing Ca^{2+} entry; however, there is currently no evidence that this alkaloid can directly block voltage-activated Ca^{2+} channels in neurones (e.g. Kawai & Watanabe, 1989). Vais *et al*. (1996) reported that ryanodine (and several other ryanoid compounds) could interfere with K^+ fluxes in locust and mouse muscle membranes, suggesting that ryanodine could exert a weak block of the putative K^+ conductance underlying the sADP; if this were the case, then one would expect to see a small depolarization during ryanodine superfusion. Since the observed reduction of I_{ADP} was very small, any effects of altering conductance through this type of channel would probably be masked by other background leak currents present, making such a depolarization difficult to detect.

The possible involvement of inositol 1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} stores (Henzi & MacDermott, 1992) in I_{ADP} generation would have been difficult to assess, as there are currently no membrane permeant agents available which affect these sites. The use of heparin in recording pipettes, to inhibit IP_3-sensitive Ca^{2+} release (Abdul-Ghani *et al*., 1996; Li & Hatton, 1997) was considered impractical, since it was

impossible to guarantee that recordings were always being made from 'type 1' responding olfactory neurones (expressing I_{ADP}) at the outset (Libri *et al*., 1994).

Other pharmacological actions of caffeine

Caffeine is known to have a number of pharmacological actions in addition to augmenting intracellular Ca^{2+} release. Firstly, caffeine inhibits phosphodiesterase activity, thus leading to a buildup of intracellular cyclic adenosine monophosphate (cyclic AMP) within treated cells (for reviews see Nehlig *et al*., 1992; Sawynok & Yaksh, 1993). In order to examine whether this action of caffeine was responsible for I_{ADP}, inhibition, a more selective inhibitor of phosphodiesterase, IBMX, was added to the bathing solution in the presence of OXO-M. At this concentration, IBMX was expected to be a potent inhibitor of the phosphodiesterase (Beavo & Reifsnyder, 1990), while having little or no effect on intracellular calcium release *per se*, normally observed at millimolar concentrations (Usachev & Verkhratsky, 1995). However in our experiments, application of IBMX (in OXO-M) failed to reduce the amplitude of I_{ADP}, even after 20–30 min exposure; this would suggest that inhibition of phosphodiesterase (and increase of intracellular cyclic AMP) was not responsible for I_{ADP} inhibition.

It is interesting to note that IBMX can also act as an adenosine receptor antagonist at the concentration used in the present study (Schwabe *et al*., 1985; Prestwich *et al*., 1987). Considering that caffeine shares this action (Greene *et al*., 1985; Haas & Greene, 1988) it seems rather unlikely that pharmacological inhibition of endogenously-released adenosine (Motley & Collins, 1983) was involved in the effect. Furthermore, in experiments where a high concentration of adenosine was used in an attempt to overcome any possible antagonism of endogenous adenosine receptors imposed by caffeine, there was no reversal of the I_{ADP} depression, even though the e.p.s.p amplitude was clearly decreased (Figure 6D) (the latter was most likely due to activation of presynaptic (inhibitory) adenosine receptors; *c.f*. McCabe & Scholfield, 1985).

Caffeine affects calcium entry into neurones during generation of I_{ADP}

The original experiments of Constanti *et al*. (1993) clearly showed that neuronal Ca^{2+} entry was essential for the generation of I_{ADP}, insofar as removal of Ca^{2+} from the external bathing medium abolished the afterdepolarizing response. In the present study we found that 10 μM nifedipine (a potent and selective L-type calcium channel blocker; Bean, 1989; Kostyuk, 1999) inhibited the I_{ADP} by 42%, indicating a clear importance of these channels in I_{ADP} generation. However, the selective N-type calcium channel blocker ω-CTx GVIA (at 200 nM) only reduced I_{ADP} by an average of 20%, and in a highly variable fashion, suggesting that N-channels were less generally involved in I_{ADP} generation in these cells.

Since caffeine can directly interfere with inward calcium currents in muscle preparations (Zholos *et al*., 1991; Varro *et al*., 1993; Yoshino *et al*., 1996), it is conceivable that it was blocking Ca^{2+} influx through voltage-sensitive Ca^{2+} channels, and leading to an inhibition of the I_{ADP}. In recordings made in Cs^{+}-loaded cells (to block outward repolarizing K^{+} conductances; Galvan *et al*., 1985; Libri *et al*., 1996), caffeine was able to significantly reduce the half-width of the initial spike in an evoked spike train, suggesting a blockade of voltage-gated Ca^{2+} currents. For comparison, 10 μM nifedipine produced a similar degree of spike shortening as was seen in the presence of caffeine (43 and 45% inhibition in nifedipine or caffeine respectively).

In additional experiments where 1 μM nifedipine was added to cells while in the presence of 3 mM caffeine, no additive blockade of the Ca^{2+} spike plateau was observed, suggesting that caffeine and nifedipine were acting at a common site, i.e. the L-type calcium channel. Moreover, applying 3 mM caffeine in the presence of 10 μM nifedipine exerted no further reduction in the Ca^{2+} spike half-width. It is thus likely that inhibition of I_{ADP} by caffeine may partly result from a direct block of an L-type Ca^{2+} conductance similar to that affected by nifedipine. However, it is worth noting that nifedipine inhibited I_{ADP} amplitude by $\sim$42% (similar to the 43% reduction in spike half-width), whereas caffeine was able to reduce I_{ADP} amplitude by $\sim$75% (*c.f*. spike half-width inhibition of 45%). A further blocking mechanism for caffeine was therefore suggested.

Block of I_{ADP} K^{+} *conductance by caffeine?*

One further action of caffeine which could explain the residual inhibition of I_{ADP} is a direct block of the putative I_{ADP} K^{+} conductance (Constanti *et al*., 1993). Caffeine is known to block certain types of K^{+} current, without interfering with intracellular signalling mechanisms (Greene *et al*., 1985; Yamamoto *et al*., 1995); e.g. the neuronal delayed rectifier (I_{DR}) and the transient outward current (I_A) in neurones (Reiser *et al*., 1996), or I_A in smooth muscle cells (Noack *et al*., 1990). It should be noted that these currents were claimed to be independent of intracellular Ca^{2+} concentration (which must always be a consideration when testing caffeine). Interestingly, theophylline has also been shown to block a Ca^{2+}-independent K^{+} conductance in dissociated cortical neurones (Munakata & Akaike, 1993).

In conclusion, it is clear from the present experiments, that the olfactory cortical I_{ADP} tail current has a complicated mechanism of induction. It appears that Ca^{2+} entry during prolonged depolarizing stimuli takes place, at least in part, *via* L-type voltage-gated Ca^{2+} channels, and that agents capable of manipulating release of Ca^{2+} from intracellular stores have little or no effect on I_{ADP} generation. Similarly, inhibition of intracellular phosphodiesterase, or pharmacological manipulation of adenosine receptors in the slices does not affect I_{ADP}. It is therefore likely that the inhibition of I_{ADP} observed in caffeine was due in part, to a block of voltage-gated Ca^{2+} currents (reducing Ca^{2+} entry into the neurones), and also a direct block of the putative K^{+} conductance underlying the I_{ADP}. Further studies will be required to gain a full understanding of the mechanism(s) underlying this unusual afterpotential phenomenon.

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References

ABDUL-GHANI, M.A., VALIANTE, T.A., CARLEN, P.L. & PENNEFATHER, P.S. (1996). Metabotropic glutamate receptors coupled to IP_3 production mediate inhibition of I_{AHP} in rat dentate granule neurones. *J. Neurophysiol.,* **76,** 2691 – 2700.

ANDRADE, R. (1991). Cell excitation enhances muscarinic cholinergic responses in rat association cortex. *Brain Res.,* **548,** 81 – 93.

ARANEDA, R. & ANDRADE, R. (1991). 5-Hydroxytryptamine$_2$ and 5-hydroxytryptamine $_{1A}$ receptors mediate opposing responses on membrane excitability in rat association cortex. *Neuroscience*, **40,** 399 – 412.

BEAN, B.P. (1989). Classes of calcium channels in vertebrate cells. *Annu. Rev. Physiol.,* **51,** 367 – 384.

BEAVO, J.A. & REIFSNYDER, D.H. (1990). Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. *Trends. Pharmacol. Sci.,* **11,** 150 – 155.

CERNE, R. & SPAIN, W.J. (1997). $GABA_A$ mediated afterdepolarization in pyramidal neurons from rat neocortex. *J. Neurophysiol.,* **77,** 1039 – 1045.

CONNORS, B.W. & GUTNICK, M.J. (1990). Intrinsic firing patterns of diverse neocortical neurons. *Trends Neurosci.,* **13,** 99 – 104.

CONSTANTI, A., BAGETTA, G. & LIBRI, V. (1993). Persistent muscarinic excitation in guinea-pig olfactory cortical neurones: involvement of a slow post-stimulus afterdepolarizing current. *Neuroscience,* **56,** 887 – 904.

CONSTANTI, A. & BAGETTA, G. (1991). Muscarinic receptor activation induces a prolonged post-stimulus afterdepolarization with a conductance decrease in guinea-pig olfactory cortex neurones *in vitro*. *Neurosci. Lett.,* **131,** 27 – 32.

COSTA, P.F., RIBEIRO, M.A., & SANTOS, A.I. (1991). Afterpotential characteristics and firing patterns in maturing rat hippocampal CA1 neurons in in vitro slices. *Brain Res. Dev. Brain Res.,* **62,** 263 – 272.

DALY, J.W., BRUNS, R.F. & SNYDER, S.H. (1981). Adenosine receptors in the central nervous system: relationship to the central actions of methylxanthines. *Life Sci.,* **28,** 2083 – 2097.

ENDO, M. (1977). Calcium release from the sarcoplasmic reticulum. *Physiol. Rev.,* **57,** 71 – 108.

FREDHOLM, B.B., BÄTTIG, K., HOLMÉN, J., NEHLIG, A. & ZVARTAU, E.E. (1999). Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol. Rev.,* **51,** 83 – 133.

GALVAN, M., CONSTANTI, A. & FRANZ, P. (1985). Calcium-dependent action potentials in guinea-pig olfactory cortex neurones. *Pflügers Arch.,* **404,** 252 – 258.

GREENE, R.W., HAAS, H.L. & HERMANN, A. (1985). Effects of caffeine on hippocampal pyramidal cells *in vitro*. *Br. J. Pharmacol.,* **85,** 163 – 169.

GREENE, C.C., SCHWINDT, P.C. & CRILL, W.E. (1994). Properties and ionic mechanisms of a metabotropic glutamate receptor-mediated slow afterdepolarization in neocortical neurons. *J. Neurophysiol.,* **72,** 693 – 704.

GROSFILS, K., METIOUI, M., LENOBLE, S. & DEHAYE, J.P. (1996). Inhibitory effect of caffeine on response to carbachol in rat pancreatic acini. *Gen. Pharmacol.,* **27,** 1041 – 1046.

HAAS, H.L. & GREENE, R.W. (1984). Adenosine enhances after-hyperpolarization and accommodation in hippocampal pyramidal cells. *Pflügers Arch.,* **402,** 244 – 247.

HAAS, H.L. & GREENE, R.W. (1988). Endogenous adenosine inhibits hippocampal CA1 neurones: further evidence from extra- and intracellular recording. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **337,** 561 – 565.

HAJ-DAHMANE, S. & ANDRADE, R. (1997). Calcium-activated cation non-selective current contributes to the fast afterdepolarization in rat prefrontal cortex neurones. *J. Neurophysiol.,* **78,** 1983 – 1989.

HAJ-DAHMANE, S. & ANDRADE, R. (1998). Ionic mechanism of the slow afterdepolarization induced by muscarinic receptor activation in rat prefrontal cortex. *J. Neurophysiol.,* **80,** 1197 – 1210.

HENZI, V. & MACDERMOTT, A.B. (1992). Characteristics and function of Ca^{2+}- and inositol 1,4,5-trisphosphate-releasable stores of Ca^{2+} in neurons. *Neuroscience,* **46,** 251 – 273.

HIGASHI, H., TANAKA, E., INOKUCHI, H. & NISHI, S. (1993). Ionic mechanisms underlying the depolarizing and hyperpolarizing afterpotentials of single spike in guinea-pig cingulate cortical neurones. *Neuroscience,* **55,** 129 – 138.

KAWAI, T. & WATANABE, M. (1989). Effects of ryanodine on the spike after hyperpolarization in sympathetic neurones of the rat superior cervical ganglion. *Pflügers Arch.,* **413,** 470 – 475.

KOSTYUK, P.G. (1999). Low-voltage activated calcium channels: achievements and problems. *Neurosci.,* **92,** 1157 – 1163.

KUBA, K. (1994). Ca^{2+}-induced Ca^{2+} release in neurones. *Jpn. J. Physiol.,* **44,** 613 – 650.

LI, Z., DECAVEL, C. & HATTON, G.I. (1995). Calbindin-D_{28K}: role in determining intrinsically generated firing patterns in rat supraoptic neurones. *J. Physiol.,* **488,** 601 – 608.

LI, Z. & HATTON, G.I. (1997). Ca^{2+} release from internal stores: role in generating depolarizing after-potentials in rat supraoptic neurones. *J. Physiol.,* **498,** 339 – 350.

LIBRI, V., CONSTANTI, A., CALAMINICI, M. & NISTICÓ, G. (1994). A comparison of the muscarinic response and morphological properties of identified cells in the guinea-pig olfactory cortex *in vitro*. *Neuroscience,* **59,** 331 – 347.

LIBRI, V., CONSTANTI, A., ZIBETTI, M. & NISTICÓ, G. (1996). Effects of felbamate on muscarinic and metabotropic-glutamate agonist-mediated responses and magnesium-free or 4-aminopyridine-induced epileptiform activity in guinea pig olfactory cortex neurons in vitro. *J. Pharmacol. Exp. Ther.,* **277,** 1759 – 1769.

LIBRI, V., CONSTANTI, A., ZIBETTI, M. & POSTLETHWAITE, M. (1997). Metabotripic glutamate receptor subtypes mediating slow inward tail current (I_{ADP}) induction and inhibition of synaptic transmission in olfactory cortical neurones. *Br. J. Pharmacol.,* **120,** 1083 – 1095.

MAGISTRETTI, J., BREVI, S. & DE CURTIS, M. (1999). Biophysical and pharmacological diversity of high-voltage-activated calcium currents in layer II neurones of guinea-pig piriform cortex. *J. Physiol.,* **518,** 705 – 720.

MARKRAM, H., HELM, P.J. & SAKMANN, B. (1995). Dendritic calcium transients evoked by single back-propagating action potentials in rat neocortical pyramidal neurons. *J. Physiol.,* **485,** 1 – 20.

MARRION, N.V. & ADAMS, P.R. (1992). Release of intracellular calcium and modulation of membrane currents by caffeine in bull-frog sympathetic neurones. *J. Physiol.,* **445,** 515 – 535.

MCCABE, J. & SCHOLFIELD, C.N. (1985). Adenosine-induced depression of synaptic transmission in the isolated olfactory cortex: receptor identification. *Pflügers Arch.,* **403,** 141 – 145.

MOTLEY, S.J. & COLLINS, G.G.S. (1983). Endogenous adenosine inhibits excitatory transmission in the rat olfactory cortex slice. *Neuropharmacology,* **22,** 1081 – 1086.

MUNAKATA, M. & AKAIKE, N. (1993). Theophylline affects three different potassium currents in dissociated rat cortical neurones. *J. Physiol.,* **471,** 599 – 616.

NEHLIG, A., DAVAL, J.L. & DEBRY, G. (1992). Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effects. *Brain Res. Rev.,* **17,** 139 – 170.

NOACK, T.H., DEITMER, P. & GOLENHOFEN, K. (1990). Features of a calcium independent, caffeine sensitive outward current in single smooth muscle cells from guinea-pig portal vein. *Pflügers Arch.,* **416,** 467 – 469.

NOHMI, M., HUA, S.Y. & KUBA, K. (1992). Basal Ca^{2+} and the oscillation of Ca^{2+} in caffeine-treated bullfrog sympathetic neurones. *J. Physiol.,* **450,** 513 – 528.

PENNEFATHER, P., LANCASTER, B., ADAMS, P.R. & NICOLL, R.A. (1985). Two distinct Ca-dependent K currents in bullfrog sympathetic ganglion cells. *Proc. Natl. Acad. Sci. U.S.A.,* **82,** 3040 – 3044.

POSTLETHWAITE, M., CONSTANTI, A. & LIBRI, V. (1999). Caffeine blocks the post-stimulus afterdepolarizing tail current (I_{ADP}) induced by muscarinic receptor activation in olfactory cortical neurones *in vitro*. *Br. J. Pharmacol.,* **126,** 292P.

PRESTWICH, S.A., FORDA, S.R. & DOLPHIN, A.C. (1987). Adenosine antagonists increase spontaneous and evoked transmitter release from neuronal cells in culture. *Brain Res.,* **405,** 130 – 139.

REISER, M.A., D'SOUZA, T. & DRYER, S.E. (1996). Effects of caffeine and 3-isobutyl-1 methylxanthine on voltage-activated potassium currents in vertebrate neurones and secretory cells. *Br. J. Pharmacol.,* **118,** 2145 – 2151.

SAH, P. & MCLACHLAN, E.M. (1991). Ca^{2+}-activated K^+ currents underlying the afterhyperpolarization in guinea-pig vagal neurones: a role for Ca^{2+}-activated Ca^{2+} release. *Neuron,* **7,** 257 – 264.

SAWYNOK, J. & YAKSH, T.L. (1993). Caffeine as an analgesic adjuvant: a review of pharmacology and mechanisms of action. *Pharmacol. Rev.,* **45,** 43 – 85.

SCHWABE, U., UKENA, D. & LOHSE, M.J. (1985). Xanthine derivatives as antagonists at A_1 and A_2 adenosine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **330,** 212 – 221.

SCHWINDT, P.C., SPAIN, W.J., FOEHRING, R.C., CHUBB, M.C. & CRILL, W.E. (1988). Slow conductances in neurones from cat sensorimotor cortex in vitro and their role in slow excitability changes. *J. Neurophysiol.,* **59,** 450 – 467.

SMITH, S.J., MCDERMOTT, A.B. & WEIGHT, F.F. (1983). Detection of intracellular Ca^{2+} transients in sympathetic neurones using arsenazo III. *Nature,* **304,** 350 – 352.

TANABE, M., GäHWILER, B.T. & GERBER, U. (1998). L-type calcium channels mediate the slow Ca^{2+}-dependent afterhyperpolarization current in rat CA3 pyramidal cells *in vitro*. *J. Neurophysiol.,* **80,** 2268 – 2273.

TORRES, G.E., ARFKEN, C.L. & ANDRADE, R. (1996). 5-hydroxytryptamine$_4$ receptors reduce afterhyperpolarization in hippocampus by inhibiting calcium-induced calcium release. *Mol. Pharmacol.,* **50,** 1316 – 1322.

USACHEV, Y. & VERKHRATSKY, A. (1995). IMBX induces calcium release from intracellular stores in rat sensory neurones. *Cell Calcium,* **17,** 197 – 206.

VAIS, H., RUCAREANU, C. & USHERWOOD, P.N.R. (1996). Ryanoids change the permeability of potassium channels of locust (Schistocerca gregaria) muscle. *Pflügers Arch.,* **432,** 700 – 707.

VARRO, A., HESTER, S. & PAPP, J.G. (1993). Caffeine-induced decrease in the inward rectifier potassium and the inward calcium currents in rat ventricular myocytes. *Br. J. Pharmacol.,* **109,** 895 – 897.

WHITE, G., LOVINGER, D.M. & WEIGHT, F.F. (1989). Transient low-threshold Ca^{2+} current triggers burst firing through an after-hyperpolarizing potential in an adult mammalian neuron. *Proc. Natl. Acad. Sci. U.S.A.,* **86,** 6802 – 6806.

YAMAMOTO, T., KAKEHATA, S., YAMADA, T., SAITO, H. & AKAIKE, N. (1995). Caffeine rapidly decreases potassium conductance of dissociated outer hair cells of guinea-pig cochlea. *Brain Res.,* **677,** 89 – 96.

YOSHINO, M., MATSUFUJI, Y. & YABU, H. (1996). Voltage-dependent suppression of calcium current by caffeine in single smooth muscle cells of the guinea-pig urinary bladder. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **353,** 334 – 341.

YOSHIZAKI, K., HOSHINO, T., SATO, M., KOYANO, H., NOHMI, M., HUA, S. & KUBA, K. (1995). Ca^{2+}-induced Ca^{2+} release and its activation in response to a single action potential in rabbit otic ganglion cells. *J. Physiol.,* **486,** 177 – 187.

ZHOLOS, A.V., BAIDAN, L.V. & SHUBA, M.F. (1991). The inhibitory action of caffeine on calcium currents in isolated intestinal smooth muscle cells. *Pflügers Arch.,* **419,** 267 – 273.

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