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G-protein-mediated desensitization of metabotropic glutamatergic and muscarinic responses in CA3 cells in rat hippocampus

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1. Desensitization of a metabotropic response was investigated in CA3 pyramidal neurons in hippocampal slice cultures using the patch-clamp technique.

2. 1*S*,3*R*-1-aminocyclopentane-1,3-dicarboxylate (1*S*,3*R*-ACPD), an agonist at metabotropic glutamate receptors (mGluRs), and methacholine (MCh), an agonist at muscarinic receptors, induced a cationic current that appears to be activated through a G-protein-independent transduction process, as previously shown. Prolonged or repetitive bath application of agonists led to rapid desensitization of the cationic current with a time constant of ~20 s.

3. Complete recovery from desensitization was observed within 6 min.

4. These responses mediated by mGluRs and muscarinic receptors cross-desensitized.

5. Preventing the activation of G-proteins by loading cells with GDPβS strongly reduced or suppressed desensitization, and resulted in a sustained inward cationic current. When cells were filled with GTPγS to irreversibly activate G-proteins, the desensitization process was enhanced such that a first application of agonist caused a markedly reduced response.

6. These results show that a cationic current induced by metabotropic agonists in hippocampal pyramidal cells undergoes apparent desensitization and suggests that this process occurs through a G-protein-mediated inhibition of the underlying membrane conductance.

Response adaptation in neural systems is a fundamental property contributing to the maintenance of homeostasis. At the neuronal level, this regulatory mechanism is manifested as a process of desensitization in response to prolonged or repeated stimulation by neurotransmitter. Membrane receptors, the initial transducers of extracellular signals, are strategically situated to modulate cellular responsiveness by acting as components of negative feedback systems. Both ligand-gated receptors and G-protein-coupled receptors are substrates for protein kinase-mediated phosphorylation resulting in rapid desensitization (Huganir & Greengard, 1990). Metabotropic glutamate receptors (mGluRs), as members of the super-family of G-protein-coupled receptors, may undergo a similar process of desensitization. Indeed, the activation of protein kinase C using phorbol esters attenuates biochemical responses mediated by mGluRs suggesting that these receptors desensitize in response to phosphorylation (Canonico, Favit, Catania & Nicoletti, 1988; Schoepp & Johnson, 1988; Thomsen, Mulvihill, Haldeman, Pickering, Hampson & Suzdak, 1993; Herrero, Miras-Portugal & Sánchez-Prieto, 1994). Furthermore, in clonal BHK cells transfected with the mGluR subtype 1α, brief exposure to agonists resulted in a rapid and transient phosphorylation of the receptor that was prevented in the presence of a PKC inhibitor (Alaluf, Mulvihill & McIlhinney, 1995). However, in the case of electrophysiological actions mediated by mGluRs, a diminished response may involve the receptors, the G-proteins, the enzymes constituting the second messenger systems, or the ion channels as the final effectors in the signalling cascade. Components at each of these levels have been shown to exhibit consensus sequences for phosphorylation by protein kinases (for review, see Houslay, 1991) and consequently represent targets for functional downregulation.

Muscarinic cholinergic responses, which frequently show striking parallels to electrophysiological responses induced by mGluRs in many brain areas (Charpak, Gähwiler, Do & Knöpfel, 1990; Guérineau, Gähwiler & Gerber, 1994; Guérineau, Bossu, Gähwiler & Gerber, 1995*a*), have been shown to desensitize in cardiac cells, where the underlying mechanisms have recently been clarified. In atrial myocytes, acetylcholine binds to an M_2 muscarinic receptor to activate a K^+ current that rapidly desensitizes through a process

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involving membrane-associated protein kinases and cytosolic phosphatases (Kim, 1993). In cells dialysed with guanosine triphosphate (GTP), fading of the K^+ current response was reduced indicating a role for G-proteins (Kurachi, Nakajima & Sugimoto, 1987; Mubagwa, Gilbert & Pappano, 1994). A G-protein-coupled receptor kinase (GRK2) has now been identified as mediating this desensitization through phosphorylation of the muscarinic receptor (Shui, Boyett, Zang, Haga & Kameyama, 1995).

The purpose of this study was to characterize the desensitization of a cationic current induced by activation of metabotropic glutamatergic or muscarinic receptors. It was previously shown that this cationic current does not appear to be mediated by G-proteins, in the sense that interruption of G-protein function did not prevent the induction or diminish the amplitude of the response (Guérineau *et al.* 1995*a*). In this investigation, however, evidence is presented that G-proteins are involved in the rapid desensitization of the response following prolonged or repetitive exposure to agonists. A preliminary account of these results has been presented in abstract form (Guérineau, Bossu, Gähwiler & Gerber, 1995*b*).

METHODS

Hippocampal slice cultures

Experiments were performed using organotypic hippocampal slice cultures prepared as described previously (Gähwiler, 1981). Hippocampi were removed aseptically from 6-day-old Wistar rats which had been killed by decapitation following cervical dislocation. The nutrient medium was composed of 50% Eagle's basal medium, 25% Hanks' or Earle's balanced salt solution, and 25% heat-inactivated horse serum, supplemented with glucose to a final concentration of 6·5 mg ml^{-1}. After 15–30 days *in vitro*, slices were transferred to a recording chamber attached to the stage of an inverted microscope fitted with differential interference contrast optics and continuously superfused with an extracellular solution at 25 °C containing (mM): 124 NaCl, 16 KCl, 1·8 $CaCl_2$, 2 $MgCl_2$, 11·6 $NaHCO_3$, 0·4 NaH_2PO_4 and 5·6 glucose, which was buffered to pH 7·3 by bubbling with 95% O_2–5% CO_2. Propagated electrical transmission was blocked by adding tetrodotoxin (TTX, 0·5 μM) to the superfusing solution.

Electrophysiological recordings

Recordings were obtained from CA3 pyramidal neurons, using the patch-clamp technique in the whole-cell configuration. Patch pipettes were pulled to a resistance of 2–4 MΩ and filled with the following solution (mM): 140 potassium gluconate, 10 KCl, 2 $MgCl_2$, 1·1 EGTA and 5 Hepes; adjusted to pH 7·2 with KOH. In some experiments, cells were loaded with GTPγS (250–500 μM) or GDPβS (1 mM) by passive diffusion from patch pipettes for 10–15 min. Membrane currents were recorded under voltage-clamp conditions using a List EPC-7 amplifier (List Electronic). High resistance seals (> 1 GΩ) were obtained by gently advancing pipettes against the membrane of visually identified CA3 cells and then applying negative pressure. The membrane beneath the pipette was then ruptured with strong negative pressure to achieve continuity with the intracellular milieu. Cells were discarded if the access resistance was > 10 MΩ, to ensure adequate intracellular dialysis. Patch-clamp signals were fed into separate channels of an analog to digital converter (TL-1/DMA interface, Axon Instruments), digitized, stored and analysed on a PC using pCLAMP software. Voltage pulse generation, data acquisition and analysis were performed with the same software/hardware system.

In a few experiments, the single-electrode voltage-clamp method was used to record intracellularly from CA3 pyramidal neurons (Axoclamp-2 amplifier, Axon Instruments). Cells were impaled with thin-walled electrodes filled with a solution containing 2 M potassium methylsulphate (buffered to pH 7·0 with KOH), resulting in a tip resistance of 60–75 MΩ. Membrane currents were recorded in cells held at −50 mV.

Drugs

Drugs were directly applied via the superfusion solution and reached equilibrium in the recording chamber (volume 0·6 ml) in about 1 min. 1*S*,3*R*-1-aminocyclopentane-1,3-dicarboxylate (1*S*,3*R*-ACPD), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and D-2-amino-5-phosphonovalerate (D-APV) were purchased from Tocris Cookson (Bristol, UK), TTX from Sankyo Co. Ltd (Tokyo, Japan), acetyl-β-methylcholine chloride (methacholine, MCh), L-glutamate, 8-bromo-cAMP and phorbol 12,13-dibutyrate (PDBu) from Sigma, and pertussis toxin (PTX) from List Biological Laboratories (Campbell, CA, USA). (2*S*,1′*R*,2′*R*,3′*R*)-2-(2,3-dicarboxycyclopropyl)-glycine (DCG-IV), an agonist at mGluR2 and mGluR3, was a gift from Dr Y. Ohfune of the Suntory Institute for Biomedical Research (Osaka, Japan). As this compound is also a potent agonist at NMDA receptors (Ohfune, Shimamoto, Ishida & Shinozaki, 1993), experiments with DCG-IV were always done in the presence of D-APV (100 μM). The following substances were introduced into cells through passive diffusion from the patch pipette: guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS) from Boehringer Mannheim, adenosine 5′-triphosphate (ATP), and guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) from Sigma.

Statistics

Numerical data in the text and figures are expressed as means ± standard error of the mean. Student's *t* test was used to compare means when appropriate. In some experiments, differences between two groups were assessed using the non-parametric Mann–Whitney *U* test. $P < 0·05$ was considered significant.

RESULTS

Desensitization of cationic current

We have previously characterized a cationic conductance induced by stimulation of mGluRs or muscarinic cholinergic receptors that appears to activate without the involvement of a G-protein intermediary (Guérineau *et al.* 1995*a*). The desensitization properties of this response following prolonged or repetitive application of 1*S*,3*R*-ACPD (50 μM), a selective mGluR agonist, or MCh (5 μM), an agonist at cholinergic muscarinic receptors, were investigated in CA3 pyramidal neurons. For the purpose of this study, the term 'desensitization' is taken to refer to the empirical observation of a decrease in responsiveness (Tan & Marty, 1991), rather than to a specific mechanism involving a change in receptor conformation (Katz & Thesleff, 1957). To facilitate the study of the cationic conductance, experiments were conducted under conditions of raised extracellular potassium concentration (16 mM) to maximize the cationic current response (Guérineau *et al.* 1995*a*). Furthermore, neurons were clamped at −50 mV, a potential close to the

equilibrium potential for K^+ ions under our recording conditions. This was necessary because 1*S*,3*R*-ACPD and MCh also modulate a number of K^+ conductances (Charpak, Gähwiler, Do & Knöpfel 1990; Guérineau *et al.* 1994). The alternative of recording with a caesium-based patch solution was unsuitable because the reduction in K^+ conductance still induced a slow current that followed the cationic current, albeit with a reversed polarity.

As illustrated in Fig. 1*A*, the inward cationic current response underwent desensitization with a 2 min exposure to 1*S*,3*R*-ACPD (50 μM). The response desensitized completely within 1 min ($n = 12$). In view of the apparent convergence of the transduction pathways coupling mGluRs and muscarinic receptors with the cationic conductance (Guérineau *et al.* 1995*a*), we examined whether muscarinic responses induced by MCh (5 μM, 2 min) would display a similar desensitization. This was indeed the case (Fig. 1*B*, $n = 8$). The time constants of desensitization (τ) were calculated using a mono-exponential fit and were not significantly different for 1*S*,3*R*-ACPD (18·2 ± 1·3 s, $n = 12$) and MCh (20·8 ± 2·8 s, $n = 8$; $P > 0·05$).

Rapid desensitization is not a general characteristic of metabotropic responses. When cells were bathed in an external saline containing 2·7 mM K^+, the 1*S*,3*R*-ACPD-induced reduction of the leak K^+ current ($I_{K,leak}$) appears as an inward current associated with a decrease in membrane conductance (Guérineau *et al.* 1994). This response did not desensitize during prolonged application of agonist (Fig. 1, inset, $n = 4$).

To determine the rate of recovery from desensitization, neurons were repetitively exposed to agonists for 30 s with varying time intervals. This briefer duration of application, which did not appear to change the shape of the response (Fig. 2), permitted the examination of the effects of reapplying agonists at short time intervals. When cells were exposed to 1*S*,3*R*-ACPD at 3 min intervals, the amplitude of the second response was reduced to 50·5 ± 4·2% of control ($n = 9$, Fig. 2*A*). Further applications of the agonist with the same time interval caused no additional change in the response. To evaluate the physiological significance of this desensitization, the effect of repetitive application of L-glutamate, an endogenous agonist at mGluRs, was investigated in the presence of the ionotropic glutamate receptor antagonists CNQX (20 μM) and D-APV (40 μM). The rate of recovery from desensitization observed for L-glutamate (500 μM, 30 s) was equivalent to that for 1*S*,3*R*-ACPD ($n = 7$, Fig. 2*A*, right graph). The effects of repetitive bath application of MCh (5 μM, 30 s, 3 min intervals) on the amplitude of the cationic current were also tested. With the second application, the current amplitude attained 55·1 ± 3·8% of control ($n = 10$, Fig. 2*B*). The amplitude ratio between the first and the second response did not depend on the amplitude of the first response (correlation coefficient $r^2 = 0·01$ for 1*S*,3*R*-ACPD, $n = 14$, and 0·04 for MCh, $n = 16$; data not shown). With 10 min intervals between applications, the amplitude of the agonist-induced current remained constant (Fig. 3). For example, the response to the sixth application of 1*S*,3*R*-ACPD, L-glutamate, or MCh was not significantly different from the first response ($P > 0·05$; $n = 3$ for 1*S*,3*R*-ACPD and MCh, $n = 4$ for L-glutamate). Furthermore, after a protocol involving repetitive exposures to 1*S*,3*R*-ACPD or MCh every 3 min, a 10 min interval without drug application sufficed to permit full recovery of the subsequent response ($n = 7$ for 1*S*,3*R*-ACPD and $n = 5$ for MCh; data not shown). These results indicate that desensitization is unlikely to be due to an irreversible run-down phenomenon associated with the recording technique, i.e. patch clamp in the whole-cell configuration. This was confirmed in experiments using sharp microelectrodes to avoid intracellular dialysis. When 1*S*,3*R*-ACPD was applied at 3 min intervals, the ratio of the amplitude between the first and the subsequent responses again amounted to about 50% ($n = 5$; data not shown).

The time course for the recovery from desensitization was determined by plotting the ratio of the amplitude of the control *versus* subsequent responses as a function of the time interval between applications of agonists (Fig. 4). A 6 min interval between agonist applications was sufficient for full recovery. The time for 50% recovery from desensitization (t_{50}) was approximately 3 min.

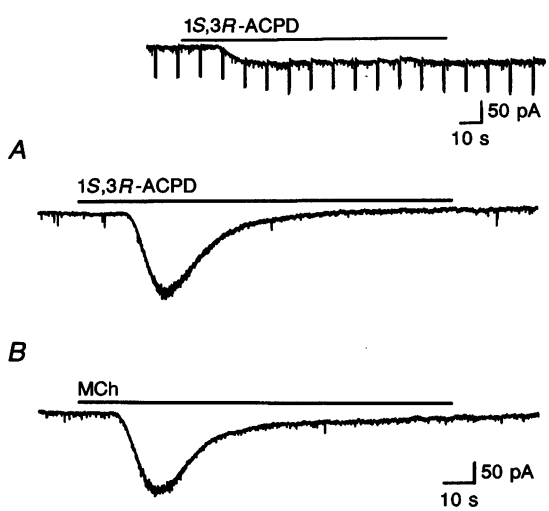

Figure 1. Desensitization of the cationic current response upon prolonged application of 1*S*,3*R*-ACPD and MCh

A, a CA3 pyramidal cell voltage clamped at −50 mV was exposed to 50 μM 1*S*,3*R*-ACPD for 2 min in the presence of 16 mM extracellular K^+. The resulting inward cationic current underwent rapid desensitization. *B*, desensitization of the current recorded in the same neuron after a 2 min application of 5 μM MCh. Inset: 1*S*,3*R*-ACPD-induced current recorded in 2·7 mM external K^+. This current, resulting from the reduction of $I_{K,leak}$, persisted as long as the agonist was perfused. Hyperpolarizing steps from −50 to −60 mV were imposed to assess changes in membrane conductance.

Cross-desensitization between metabotropic glutamatergic and muscarinic responses

Desensitization of MCh responses occurred following prior exposure to 1*S*,3*R*-ACPD, and vice versa. Figure 5 shows examples of this heterologous desensitization between responses mediated by activation of mGluRs and muscarinic receptors, in which alternating applications of the two agonists at 3 min intervals again resulted in an ~50% reduction in the amplitude of the second response ($n = 13$). The concentrations of 1*S*,3*R*-ACPD (50 μM) and MCh (5 μM) used in this study were saturating and induced cationic current responses of similar amplitude (Guérineau *et al.* 1995*a*). Thus the response to the first agonist can be taken as a control for the second response. The use of this procedure is further justified by the fact that after allowing 10 min for full recovery, the amplitude of the response to a reapplication of the second agonist was not significantly different from the control response to the first agonist ($P > 0{\cdot}05$, Fig. 5).

Desensitization is a G-protein-dependent process

Experiments were then conducted to determine whether G-proteins are involved in mediating the desensitization. As previously shown (Guérineau *et al.* 1995*a*), the cationic current induced by 1*S*,3*R*-ACPD or MCh is neither blocked by intracellular dialysis with GDPβS nor enhanced by GTPγS. Interestingly, however, in cells dialysed with a 2-fold higher concentration of GDPβS than that previously used (1 mM *versus* 500 μM, 10–15 min before agonist exposure), the cationic current was greatly increased in amplitude with the first application of 1*S*,3*R*-ACPD or MCh (to 163·1 ± 3·7% for 1*S*,3*R*-ACPD, $n = 45$, and 148·2 ± 13·2% for MCh, $n = 28$; Figs 6 and 7) compared with control conditions, and in 68% of these cells this effect was irreversible for the duration of the recording (> 10 min). In the remaining cells that did exhibit a reversible response, the recovery from desensitization of the 1*S*,3*R*-ACPD-induced response at 3 min was significantly accelerated compared with cells recorded in the absence of GDPβS (24·7 ± 5·2% desensitization *versus* 50·5 ± 4·2%, $n = 7$ and 9, respectively; $P < 0{\cdot}05$; not shown).

The loading of cells with GDPβS in the absence of agonist induced a progressive and irreversible inward current in 41% of the cells and an increase in membrane current noise never seen under control conditions, suggesting that G-proteins exert a tonic inhibition on membrane conductance. Fifteen minutes after the establishment of whole-cell recording, the inward current attained an amplitude of 254·7 ± 31·1 pA associated with an increase in conductance to 35·8 ± 1 nS (*versus* 18·4 ± 1·4 nS, $n = 25$, in control conditions). In these cells, application of 1*S*,3*R*-ACPD or MCh always induced additional irreversible inward current. To verify that the irreversible current seen in the presence of GDPβS corresponded to the cationic current under study, its reversal potential was determined by linearly increasing the

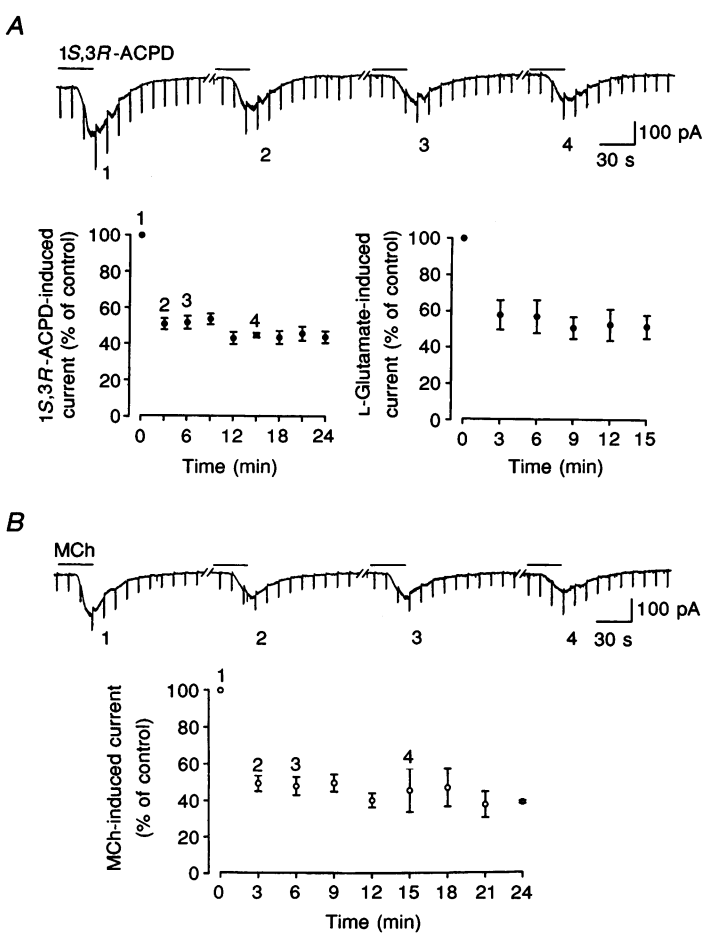

Figure 2. Partial recovery from desensitization in cells stimulated every 3 min with 1*S*,3*R*-ACPD-, L-glutamate- and MCh-induced cationic currents

A, 1*S*,3*R*-ACPD (50 μM, 30 s) was bath applied every 3 min for 24 min. A 50% recovery from desensitization was observed with the second response but further applications of the agonist caused no further effect. Hyperpolarizing steps from −50 to −60 mV were imposed to assess changes in membrane conductance. The graph on the left represents pooled data from 9 cells for 1*S*,3*R*-ACPD-induced currents. Similar results were obtained in cells stimulated with L-glutamate (0·5 mM in the presence of 20 μM CNQX and 40 μM D-APV, graph on right). *B*, a typical chart record and a graph illustrating results for 10 cells repetitively stimulated with MCh (5 μM, 30 s) at 3 min intervals.

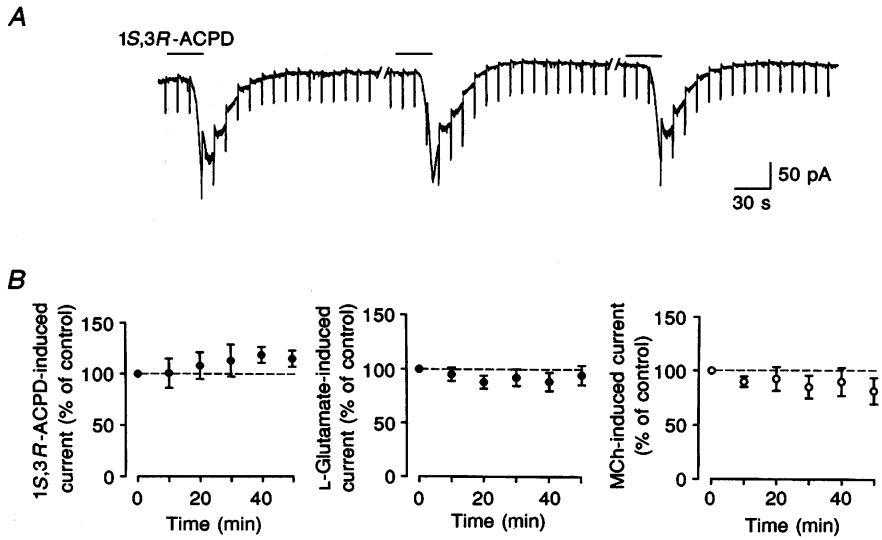

Figure 3. Full recovery from desensitization of metabotropic responses in cells stimulated every 10 min

A, chart record showing the cationic current in response to 50 μM 1*S*,3*R*-ACPD applied at 10 min intervals for 50 min in a pyramidal cell. Under these conditions, the amplitude of each response was fully recovered when compared to the first application. Hyperpolarizing steps from −50 to −60 mV were imposed to assess changes in membrane conductance. *B*, pooled data for the 3 agonists.

voltage using a ramp protocol before and during the application of the two agonists. The currents generated during the voltage ramp were well fitted by a computed linear regression ($r^2 = 0{\cdot}98$–$1{\cdot}00$), as described previously (Guérineau *et al.* 1995*a*). The extrapolated reversal potentials were $-0{\cdot}6 \pm 3{\cdot}8$ mV for 1*S*,3*R*-ACPD, $n = 8$, and $5{\cdot}0 \pm 4{\cdot}1$ mV for MCh, $n = 7$; $P > 0{\cdot}05$; data not shown). These values are close to −1·2 mV, a reversal potential expected for a non-selective cationic current, given the experimental $[\text{cation}]_i = 144{\cdot}8$ mM and $[\text{cation}]_o = 152$ mM.

Dialysis with GTPγS (250–500 μM in the patch pipette) induced a gradual outward current in twenty-one of thirty-one cells that reached a peak amplitude of $144{\cdot}8 \pm 11{\cdot}8$ pA within 10 min. In these experiments the amplitude of the response to a first agonist application was significantly decreased (to $63{\cdot}1 \pm 3{\cdot}7\%$ for 1*S*,3*R*-ACPD, $n = 26$, and $63 \pm 4{\cdot}7\%$ for MCh, $n = 25$; Figs 6 and 7). This implies that in the presence of GTPγS, the cationic conductance is already partially desensitized at the time of the first agonist application. To determine the G-protein subtype involved, hippocampal slice cultures were pretreated with pertussis toxin (PTX, 500 ng ml^{-1} for 48–72 h), which inactivates G-proteins of the G_i/G_o subtype (Katada & Ui, 1982). In each cell tested, the efficacy of PTX treatment was established by demonstrating the lack of effect of baclofen, an agonist at $GABA_B$ receptors coupled to K^+ channels via PTX-sensitive G-proteins (Andrade, Malenka & Nicoll, 1986). Under these conditions, no significant change in the responses induced by 1*S*,3*R*-ACPD or MCh was observed (Fig. 7). In addition, desensitization of the response to 1*S*,3*R*-ACPD and MCh still occurred in PTX-treated cells dialysed with GTPγS, confirming that the G-protein mediating desensitization is PTX insensitive.

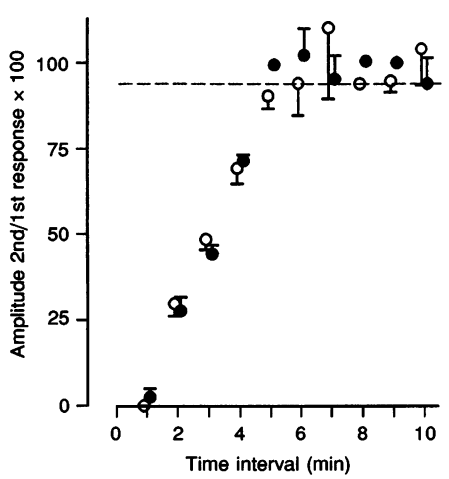

Figure 4. Time course of recovery from desensitization

Cells were consecutively stimulated with 1*S*,3*R*-ACPD (50 μM, open symbols) or MCh (5 μM, filled symbols) at different time intervals (1–10 min). Each data point illustrates the mean result for 2–17 cells. The 4 points without error bars represent results where $n = 2$ cells. For a time interval $\geqslant 5$ min between the two applications, desensitization did not occur. The time for 50% recovery from desensitization (t_{50}) is approximately 3 min.

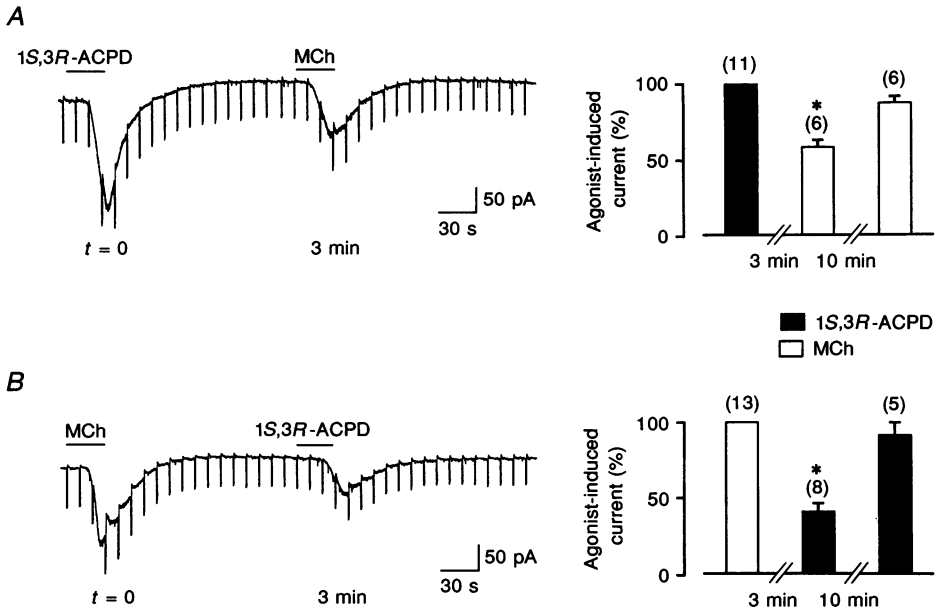

Figure 5. Heterologous desensitization in response to 1*S*,3*R*-ACPD and MCh

Cells were consecutively stimulated with a 3 min interval first with 1*S*,3*R*-ACPD and second with MCh (*A*) or vice versa (*B*). In both cases, the second response was decreased by ~50%. As shown in the histograms, the current amplitudes recovered fully after a 10 min period. The number of tested cells is indicated in parentheses. * $P < 0{\cdot}05$, as compared with control values.

One possible explanation for the finding that the induction of the cationic current appears to be G-protein independent while the desensitization process does require a G-protein, could be that two different subtypes of mGluR may be responsible for each mechanism. For example, it has been shown that the activity of L-type Ca^{2+} channels in cerebellar granule cells is enhanced by activation of mGluR1/5 and inhibited by activation of mGluR2/3 (Chavis, Shinozaki, Bockaert & Fagni, 1994; Chavis, Nooney, Bockaert, Fagni, Feltz & Bossu, 1995). Since our data are consistent with an mGluR1/5 subtype mediating the induction of the cationic current (Guérineau *et al.* 1995*a*), experiments were conducted to determine whether activation of the mGluR2/3 subtype may initiate the G-protein-dependent desensitization process. Bath application of DCG-IV (5 μM, 30 s), an agonist at mGluR2 and mGluR3 (Hayashi *et al.* 1994) did not, however, induce a cationic current (5 ± 5 pA, $n = 5$; data not shown). Moreover, the extent of recovery from desensitization of the 1*S*,3*R*-ACPD-induced response at 3 min was not modified in DCG-IV-containing perfusate compared with control (51·6 ± 4·0%, $n = 5$, *versus* 50·5 ± 4·2%, $n = 9$; $P > 0{\cdot}05$). This result indicates that a G-protein-coupled mGluR2/3 does not contribute to the desensitization process.

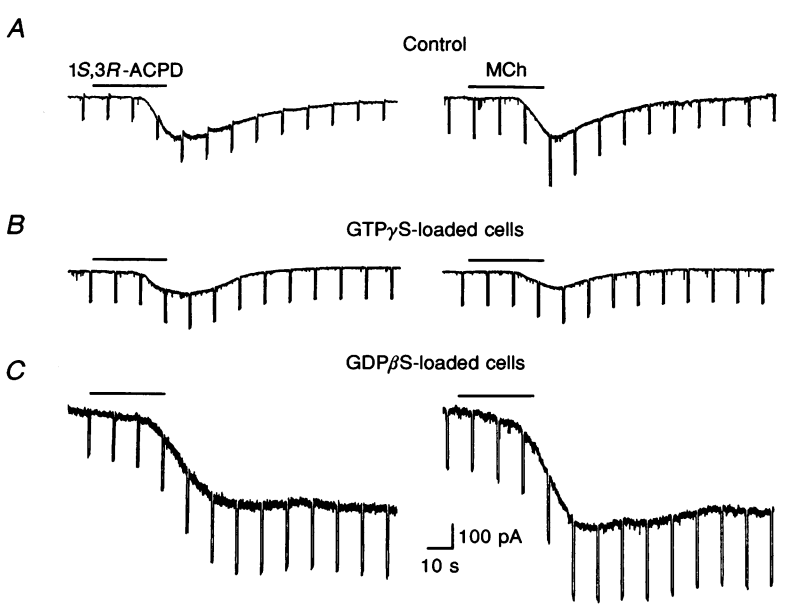

Figure 6. Inhibition of G-proteins prevents desensitization

A, typical cationic currents activated by 1*S*,3*R*-ACPD and MCh in 2 different neurons clamped at −50 mV. *B*, in GTPγS-loaded cells (500 μM for 15 min), the currents induced by both agonists were significantly reduced, as compared with the control responses. *C*, in GDPβS-loaded cells (1 mM for 15 min), the currents activated by both agonists were greatly enhanced and became irreversible.

Activation of protein kinase A or protein kinase C does not modify desensitization

Phosphorylation of membrane receptors is a major mechanism controlling the desensitization of cellular responses (Huganir & Greengard, 1990). Furthermore, mGluRs and muscarinic receptors expressed in hippocampal neurons can couple to a variety of second messenger systems that induce protein phosphorylation (Hulme, Birdsall & Buckley, 1990; Pin & Duvoisin, 1995). To examine whether a process involving phosphorylation could account for the desensitization of the cationic current, the effects of substances that activate protein kinase A and C were tested. The intracellular concentration of ATP was increased by loading cells with ATP-Mg (4 mM in the patch pipette for 10–15 min), to promote protein kinase A-dependent phosphorylation. Under these experimental conditions, recovery from desensitization was not altered ($n = 8$; data not shown). Bath application of 8-bromo-cAMP (1 mM, 3–5 min prior to agonist application), a permeable analogue of cAMP that activates protein kinase A, similarly was without effect ($n = 11$; data not shown). In cultures treated with the phorbol ester PDBu (500 nM for 3–5 min), which irreversibly activates protein kinase C, recovery from desensitization of 1*S*,3*R*-ACPD- and MCh-induced responses was not changed ($n = 4$; data not shown). Furthermore, loading cells with BAPTA, a Ca^{2+} chelator (20 mM in the patch pipette for 10–15 min prior to agonist application, $n = 8$; data not shown), had no effect on recovery from desensitization, ruling out a mechanism depending on an increase in intracellular Ca^{2+} concentration. At the above concentrations, PDBu or 8-bromo-cAMP completely blocked or significantly decreased the amplitude of the slow after-hyperpolarizing current induced by a depolarizing voltage step command. These results suggest that phosphorylation involving protein kinase A or C probably does not contribute to the recovery from desensitization.

DISCUSSION

A cationic current induced by activation of mGluRs and muscarinic cholinergic receptors in CA3 pyramidal cells undergoes complete desensitization within 1 min in the continuous presence of neurotransmitter. Transient electrophysiological responses mediated by neuronal mGluRs that may reflect desensitization have been previously observed but not further characterized (Staub, Vranesic & Knöpfel, 1992; McBain, DiChiara & Kauer, 1994; Shirasaki, Harata & Akaike, 1994; Poncer, Shinozaki & Miles, 1995). A number of other responses induced by stimulation of mGluRs, such as the reduction in a leak K^+ conductance, did not fade with prolonged agonist application (Fig. 1). For membrane currents gated by muscarinic cholinergic receptors the process of desensitization is better understood. In particular the fading of the muscarinic K^+ current ($I_{K(ACh)}$) in atrial myocytes has been extensively investigated and has recently been shown to occur mainly through the G-protein-dependent phosphorylation of the membrane receptors (Shui *et al.* 1995). In the case of the cationic current in the present study, however, an alternative mechanism not involving receptor phosphorylation may mediate the desensitization process as discussed below.

The reduction in the cationic current amplitude with repetitive stimulation is not due to a run-down phenomenon associated with the whole-cell patch-clamp recording method since (1) the reduction in the responses was reversible, and (2) desensitization was also observed with the intracellular recording technique where loss of cytosolic components by diffusion is minimized.

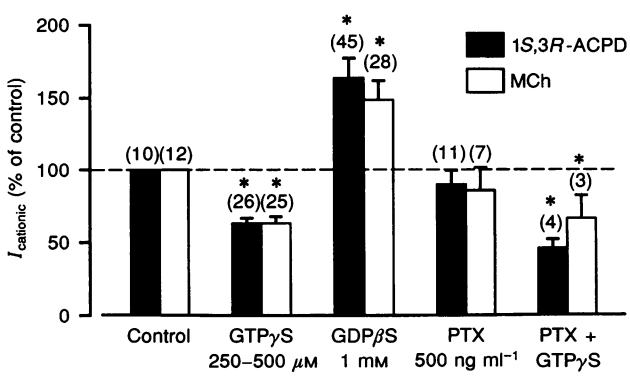

Figure 7. Summary of the effects of manipulating G-protein function on the desensitization process

The effects of G-protein activators and inhibitors on the amplitude of the cationic current induced by 1*S*,3*R*-ACPD or MCh were examined. In the presence of GTPγS in the patch pipette (250–500 μM for 10–15 min), the currents activated by the two agonists were significantly reduced (to 63·1 ± 3·7% for 1*S*,3*R*-ACPD, $n = 26$; 63 ± 4·7 for MCh, $n = 25$), whereas dialysis with GDPβS strongly potentiated responses (to 163·1 ± 3·7% for 1*S*,3*R*-ACPD, $n = 45$; 148·2 ± 13·2 for MCh, $n = 28$). Pertussis toxin pre-treatment (PTX, 500 ng ml^{-1} for 48 h) did not modify the response either in control conditions or in the presence of GTPγS. The number of cells tested at each experimental condition is indicated in parentheses. * $P < 0·05$, as compared with control values.

Time course for desensitization and recovery from desensitization

Desensitization has been reported for metabotropic responses induced by activation of glutamate receptors coupled to phospholipase C. As was the case with the cationic current, desensitization of mGluR-mediated diacylglycerol production in cortical synaptosomes occurred within 1 min (Herrero *et al.* 1994) and recovery from desensitization was established within 10–20 min. Comparable values have been determined for responses mediated by phospholipase C-coupled muscarinic receptors in rat lacrimal glands (Tan & Marty, 1991). In contrast, the hydrolysis of inositol phosphates in response to stimulation of mGluRs in cerebellar granule cells desensitized with a time constant of 30 min and persisted for up to 48 h (Catania, Aronica, Sortino, Canonico & Nicoletti, 1991). This disparity probably indicates that different intracellular transduction mechanisms are involved in the mediation of these responses.

Does desensitization occur at the level of the receptor?

Theoretically, any of the components required for the generation of the response, from the membrane receptors to the ionic channels, could be involved in the desensitization process. The desensitization of electrophysiological responses is usually initiated with the protein kinase-dependent phosphorylation of the neurotransmitter receptors (Huganir & Greengard, 1990). This is also the case for muscarinic cholinergic responses where the mechanism underlying the fade of $I_{K(ACh)}$ in atrial myocytes has been shown to involve receptor phosphorylation (Kurachi *et al.* 1987; Kim, 1993; Mubagwa *et al.* 1994; Shui *et al.* 1995). In the present case, however, the desensitization of the cationic current response may be occurring downstream to the membrane receptors. Following an initial stimulation of mGluRs, the subsequent response to muscarinic receptor stimulation was desensitized, even though these receptors had never been exposed to agonist. The same was observed when first muscarinic receptors and then mGluRs were stimulated. Previous electrophysiological studies have shown that such heterologous desensitization does not occur through receptor phosphorylation, but rather involves the modulation of subsequent components in the signal transduction pathway controlling the activity of ionic membrane channels (Kurachi *et al.* 1987; Nah, Attali & Vogel, 1993; Mubagwa *et al.* 1994).

A G-protein-dependent process

The absence of fading of the cationic current response when G-protein activation was prevented by filling cells with GDPβS implicates a G-protein-dependent mechanism in the desensitization process. Furthermore, the amplitude of the cationic current induced by a first application of agonist was dependent on the state of activation of the G-proteins. When cells were filled with GDPβS, the amplitude of responses was significantly increased, whereas stimulation of G-proteins with GTPγS led to a reduction in response amplitude. Thus, G-protein-mediated desensitization exerts an inhibitory effect on the generation of the cationic current. The inhibition of G-protein activation with GDPβS is concentration dependent. GDPβS in the patch pipette at 1 mM was required to modulate the desensitization process. In contrast, with 500 μM GDPβS, which completely blocks the effects of 1*S*,3*R*-ACPD or MCh on the leak potassium current, no apparent action on the amplitude of the cationic current was observed (Guérineau *et al.* 1995*a*). The diffusion of GDPβS or GTPγS into cells in the absence of agonist also induced slowly developing currents. In the case of GDPβS, the inward current associated with an increase in membrane conductance may reflect a slow exchange of GDP for GDPβS during the GDP–GTP binding cycle resulting in an irreversible relief of a tonic inhibitory action mediated by G-proteins on the cationic channel.

Recently, a G-protein-dependent mechanism of desensitization has also been reported for NMDA-activated currents where activation of G-proteins reduced the recovery from a glycine-independent form of desensitization (Turecek, Vyklický, Vlachová & Vyklický, 1995). Furthermore, a tonic inhibitory regulation of ionic channels directly by G-proteins has been reported for Ca^{2+} currents (Haws, Slesinger & Lansman, 1993; Boland & Bean, 1993; Netzer, Pflimlin & Trube, 1994; Elhamdani, Bossu & Feltz, 1995). A similar process may underly our observation of a slow inward current associated with an increase in membrane conductance in response to diffusion of GDPβS into cells in the absence of agonist.

Different mechanisms underlie induction and desensitization of cationic current

The fact that disruption of G-protein-mediated intracellular signalling enhanced rather than diminished the magnitude of the cationic current provides additional support for our conclusion that the induction of this current is unlikely to involve G-protein activation (Guérineau *et al.* 1995*a*). Although inhibition of an inhibitory G-protein would lead to response enhancement, it is unlikely that the mGluR mediating the cationic response is associated with an inhibitory G-protein such as G_i. Downregulation of G_i/G_o with pertussis toxin treatment was without effect, and DCG-IV, an agonist selective for class II mGluRs coupled to G_i (Hayashi *et al.* 1994), neither activated the cationic current nor modified the characteristics of desensitization.

Considering that we have not as yet found evidence for the involvement of a second messenger transduction pathway, it is possible that the desensitization of cationic current occurs through a direct interaction between G-proteins and ionic channels. An alternative hypothesis that could explain our results would be that G-proteins act at the level of the receptor to modulate agonist binding. According to this scheme, the agonist–receptor complex would be stabilized by GDP while the free receptor would be stabilized by GTP, such that a G-protein-mediated mechanism induces deactivation rather than desensitization of the response.

Recently, it has been reported that mGluRs and muscarinic cholinergic receptors mediate oscillatory behaviour consisting

of synchronized bursts of activity in hippocampal neuronal networks (Bianchi & Wong, 1994, 1995). Desensitization of the excitatory response may contribute in initiating the waning phase of the cycle and thereby in setting the rate of bursting. This could play a role both in physiological functions and in disorders such as epilepsy, where stimulation of mGluRs has been found to increase the frequency of epileptiform bursting in CA3 cells (Merlin, Taylor & Wong, 1995).

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