Order of Application Determines the Interaction Between Phorbol Esters and GTP-γ-S in Dorsal Raphe Neurons: Evidence That the Effect of 5-HT Is Modified Upstream of the G Protein Ca Channel Interaction

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Chen, Yuan and Nicholas J. Penington. Order of application determines the interaction between phorbol esters and GTP-γ-S in dorsal raphe neurons: evidence that the effect of 5-HT is modified upstream of the G protein Ca channel interaction. J. Neurophysiol. 77: 2697–2703, 1997. Phorbol esters activating protein kinase C (PKC) partially uncouple the inhibitory effect of serotonin (5-HT) from serotonergic neuron Ca^{2+} current. Presently the site of action of PKC is not known and may be the receptor, G protein, or ion channel. We recorded Ca^{2+} current from acutely isolated neurons with the use of the patch-clamp technique to study the site of action of PKC. Activation of the G protein with internal guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S) occluded the response to 5-HT, but unexpectedly this effect was not reversed by the addition of the phorbol ester phorbol 12-myristate 13-acetate (PMA) despite the voltage-dependent reversal of the effect of GTP-γ-S by long depolarizing steps to +80 mV. PMA was, however, able to partially reverse 5-HT-induced inhibition of Ca^{2+} current. The rate of reactivation of Ca^{2+} current (related to the concentration of activated G proteins) by GTP-γ-S after the addition of PMA at −50 mV was identical to the rate when only GTP-γ-S was present. By contrast, when cells were exposed first to PMA, and then GTP-γ-S was perfused into the cell, GTP-γ-S lost about half of its ability to activate the G protein. The rate of reactivation of the Ca^{2+} current by internal GTP-γ-S was also reduced in cells pretreated with PMA. The original result in which PMA did not reverse the action of GTP-γ-S suggested that the channel was not the functional site of action of PMA, nor was the site on the G protein that binds to the channel, but it did not rule out the receptor. When the receptor was bypassed, after prior PKC activation, it was found that direct activation of the G protein by a nonhydrolyzable analogue of GTP was reduced; taken as a whole, this indicates that in dorsal raphe, and perhaps other neurons, the site of the critical phosphorylation may be on the G protein and possibly at the GTP binding site.

INTRODUCTION

A possible role for protein kinase C (PKC) in the regulation of the inhibitory modulation of neurotransmitter effects on Ca^{2+} current in neurons has been described (Chen and Penington 1996; Golard et al. 1993; Lester and Jahr 1990; Swartz 1993; Zhu and Ikeda 1994). Ca^{2+} current is consistently enhanced in some neurons as a result of stimulation of PKC by phorbol esters such as phorbol 12-myristate 13-acetate (PMA) (Swartz 1993; Zhu and Ikeda 1994). In all of these studies phorbol esters disrupted neurotransmitter-activated, G-protein-mediated inhibition of Ca^{2+} current, with the notable exception of a study in frog sympathetic neurons (Yang and Tsien 1993). In some of these studies PMA decreased the effectiveness of G protein activation, irrespective of whether it was directly induced by guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S) or if it was due to receptor or tonic G-protein-mediated inhibition of Ca^{2+} current (Swartz 1993; Zhu and Ikeda 1994). It was found that the effect of GTP-γ-S on cortical cell Ca^{2+} current was reversed by the application of PMA in a voltage-dependent manner that was promoted by a 200-ms step to +80 mV every 5 s (Swartz 1993). As a result of this observation, the suggestion was made that the critical site phosphorylated by PKC may be masked by a bound activated G protein, and PKC might only be effective when the G protein was dissociated from its target (the Ca^{2+} channel), although an action of PKC at either site of interaction between the G protein and Ca^{2+} channel was considered possible (Swartz 1993). Other results were not in agreement with this scheme, despite several similarities, notably that in chick sympathetic neurons a diacylglycerol derivative capable of uncoupling transmitter effects failed to reverse the effect of GTP-γ-S (Golard et al. 1993).

It has been reported that G protein α- and β-subunits, in their inactive guanosine 5′-diphosphate (GDP)-bound form, are subject to covalent modification by PKC; thus phosphorylation may occur in a conformation-dependent manner (Rodbell 1985; Sagi-Eisenberg 1989). This effect suggests that the G protein could be a ‘‘programmable messenger’’ that after interaction with PKC would interact weakly with Ca^{2+} channels but normally with K+ channels (Chen and Penington 1996). Because of the potential light that the interaction between PMA and GTP-γ-S could shed on the site of action of PMA in dorsal raphe (DR) neurons, we tested 1) whether PMA can reverse the effect of GTP-γ-S on DR neurons; 2) whether reversing the effect of GTP-γ-S with frequent, long, and extreme depolarization would facilitate any possible reversal of the action of GTP-γ-S by PMA; and 3) (because the effects of PMA and GTP-γ-S are essentially irreversible in the time frame of an experiment) whether the order of application of the phorbol ester and GTP-γ-S is an important determinant of the ability of PMA to modify the action of GTP-
γ-S in these cells. The results of this study provide a
indication that PMA, in serotonergic DR neurons, pro-
duces its critical phosphorylation at the level of the G
protein, and furthermore at the GTP binding site and per-
haps also in other neurons where PMA does not reverse
the previously established effect of GTP-γ-S.

Methods

These results were obtained from acutely isolated serotonergic
DR neurons that exhibited stable Ba\(^{2+}\) currents of 1–3 nA in 5
mM Ba\(^{2+}\).

Cell preparation

Male Sprague-Dawley rats (200–250 g) were anesthetized with
halothane and then decapitated. Three coronal slices (500 μm)
through the brain stem at the level of the DR nucleus were prepared
with the use of a Vibroslice (Campden Instruments) in a manner
that has previously been described (Penington and Kelly 1990;
Penington et al. 1991). The slices were placed in cold artificial
cerebrospinal fluid containing (mM) 119 NaCl, 5 KCl, 2 MgCl\(_2\),
2 CaCl\(_2\), 26 NaHCO\(_3\), 1.2 NaHPO\(_4\), and 11 glucose, pH 7.3–7.4
when bubbled with 95% O\(_2\)–5% CO\(_2\). The slices were placed
on an agar base and a piece of gray matter (2 × 2 mm) was cut
from immediately below the cerebral aqueduct containing the
DR nucleus. The pieces of tissue were then incubated in a pipera-
azine-N,N’-bis(2-ethanesulfonic acid) buffer solution containing 0.07%
trypsin (Sigma Type XI) under pure oxygen for 90 min according
to the method of Kay and Wong (1987). The pieces of tissue were
then triturated in Dulbecco’s modified Eagle’s medium and the
isolated cells were allowed to settle on a glass coverslip coated
with concanavalin A. Within 5 min of plating the cells were firmly
anchored to the coated coverslip.

Recording

The extracellular solution was continually perfused at a rate of
~2 ml/min into a bath containing ~1 ml of recording solution.
Neurons with truncated dendrites and a cell soma with one dimen-
sion of ≈20 μm were voltage clamped with the use of an Axopatch
200A (Axon instruments, Foster City, CA) patch-clamp amplifier
in the whole cell configuration. Electrodes were coated with Syl-
gard and they ranged in resistance from 2 to 3 MΩ. The series
resistance of the electrode was compensated by ~80%. Leak and
capacitance were subtracted from the Ca\(^{2+}\) current records. Leak
sweeps consisted of 16 hyperpolarizing steps of 10 mV that were
then averaged. The leak sweep currents were scaled to the appro-
priate size and then subtracted from the individual current records
except where noted. Leak sweeps were obtained at regular periods
during the experiment. The voltage-clamp data were filtered at 2
kHz and then digitized at 100 μs per point. Voltage protocols were
generated and analyzed by an IBM PC 486 clone with the use of
the Axobasic 1 patch-clamp software and the resultant data were
written to disk for analysis off-line. Recordings from neurons
acutely dissociated from the adult rat brain were carried out at
room temperature (24°C). The measurements of Ca\(^{2+}\) current are
expressed as means ± SE and in some cases the differences be-
tween the means were tested for significance with the use of a
Student’s t-test.

Solutions

The pipette solution used in experiments that measured Ca\(^{2+}\)
current was composed of 130 mM tris(hydroxymethyl)amino-
methane (Tris) methanesulphonate, 10 mM ethylene glycol-bis(β-
aminooxyethyl ether)-N,N′,N′,N′-tetraacetic acid, 4 mM MgATP, 4
mM MgCl\(_2\), 300 μM GTP, and 14 mM phosphocreatine, pH ad-
justed to 7.3 with Trizma base. To establish seals for whole cell
recording, we routinely used the following solution: 135 mM NaCl,
20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid
(HEPES), 10 mM glucose, 5 mM CaCl\(_2\), 1 mM KCl, 1 mM
MgCl\(_2\), and 0.1 μM tetrodotoxin. The external recording solution,
designed to isolate calcium channel currents (carried by Ba\(^{2+}\)),
contained (mM): 160 tetrathylammonium chloride (TEACl), 5
BaCl\(_2\), 10 HEPES, and 20 sucrose, pH adjusted to 7.3 with tetra-
ethylammonium hydroxide.

Serotonin (5-HT) creatine sulfate, GTP-γ-S tetralithium salt,
and PMA were obtained from Sigma Chemical (St. Louis, MO).
PMA was dissolved in dimethyl sulfoxide and diluted 10,000 times
to its final concentration. Different concentrations of 5-HT or PMA
were added to the bath near the cell with the use of an electric-
value-controlled flow pipe fast application system.

Results

Addition of PMA did not reverse the effects of GTP-γ-S
but could reverse the 5-HT-mediated inhibition of calcium current

In the absence of GTP-γ-S, responses to 5-HT were al-
ways readily reversible on washing the 5-HT from the bath.
When 30 μM of the nonhydrolyzable analogue GTP-γ-S
(Eckstein 1985) was added to the pipette to yield a ratio of
GTP-GTP-γ-S of 10:1 (Pfaffinger 1988), there was usually a
swift reduction in the size of peak Ca\(^{2+}\) channel current
even before 5-HT was added. As previously reported, this
reduction was accompanied by a slowing in the rate of acti-
vation of the inward current and the action of 5-HT was no
longer reversible (n = 6, Fig. 1). A second application of
5-HT produced only a small additional inhibition of the Ca\(^{2+}\)
current. Traces before and after the addition of 5-HT are
shown in Fig. 1A. Figure 1 is somewhat unusual in that the
kinetics of the current is not slowed immediately after going
whole cell. In subsequent experiments in which GTP-γ-S
was used, its effect was almost maximal as soon as the
current could be measured, even with a GTP-GTP-γ-S ratio of
20:1. To provide a better comparison with earlier work,
we also removed GTP from the patch pipette and exchanged
it for the same concentration (300 μM) of GTP-γ-S; the
data were identical (n = 3). Addition of 1 μM PMA did
not reverse the effect of GTP-γ-S (Fig. 1B), consistent with
an action of PMA exerted at or upstream of the activation
of the G protein. Indeed the average of the integral of inward
current before PMA was 82.0 ± 16.2 (SE) pC, and after
PMA it was 74.57 ± 19.9 pC (n = 9; 300 and 30 μM data
lumped together). There was a decrement in the Ca\(^{2+}\) current
after PMA instead of the expected increase.

In the absence of GTP-γ-S, the addition of concentrations
of 5-HT as high as 100 μM without washing it off causes
a nondesensitizing inhibition of DR Ca\(^{2+}\) current (not
shown). In Fig. 2A, 1 μM 5-HT produced a stable inhibition.
In a different cell, Fig. 2B shows that addition of 5-HT and
PMA together (both at 1 μM) caused the gradual but partial
recovery of the effect of 5-HT. When the effect of 5-HT
was washed off, subsequent 5-HT application was less effec-
tive at inhibiting the Ca\(^{2+}\) current. In six experiments PMA
caused a 49.2 ± 9.8% recovery of the effect of constant 5-
HT application.
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The absolute amount of current facilitated as a percentage of the control current in GTP-γ-S. In control conditions this measure tends to fall a little as facilitation washes out with time (Ikeda 1991); however, after addition of PMA we found no significant decrease in the facilitation of current in GTP-γ-S to current after a prepulse was added (average 81 ± 7.8%; in PMA, 72.3 ± 8.8%). Notably, in every cell after the depolarizing prepulses were given for several minutes, depolarization to +80 mV was still able to facilitate the calcium current by >50%, even in the presence of PMA, and this is in stark contrast to results obtained in other neuronal types.

Rate of reinhibition of Ca^{2+} current by activated G proteins is unchanged when PMA is added

The presumed rate of association of the G protein subunit responsible for GTP-γ-S-induced inhibition of Ba^{2+} current in the presence and absence of PMA was also measured. Previously we showed that PMA slows the rate of reinhibition of the Ca^{2+} current, after a prepulse to +80 mV, by the agonist-activated G protein (Chen and Penington 1996). The cell was held at −50 mV to inactivate T-type calcium current, and the time course of decay

FIG. 1. Phorbol 12-myristate 13-acetate (PMA) (1 μM) does not reverse direct activation of the G protein with guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S). A: current traces before (1) and after (2–4) the addition of serotonin (5-HT). Dotted line: 0 current level. B: graph of peak current against time. When 30 μM of the nonhydrolyzable analogue GTP-γ-S was added to the pipette (GTP:GTP-γ-S ratio of 10:1), 5-HT caused a reduction in the size of peak Ca^{2+} channel current and the action of 5-HT was no longer reversible. A 2nd application of 5-HT produced a small additional inhibition of the Ca^{2+} current. Addition of 1 μM of the phorbol ester PMA did not reverse the effect of GTP-γ-S.

Strong depolarization does not enable PMA to reverse the effect of GTP-γ-S in DR neurons

Swartz (1993) found that the effect of GTP-γ-S on cortical cells was reversed by the application of PMA, in a voltage-dependent manner, which required a 200-ms step to +80 mV every 5 s. When we used this protocol (Fig. 3A), PMA was still not able to reverse the effect of GTP-γ-S (300 μM). The cell was held at −50 mV to inactivate a small, 5-HT-insensitive, T-type calcium current component and the Ba^{2+} current was integrated with or without a prepulse to +80 mV. The data are plotted against time in Fig. 3B. If PMA was able to reverse the effect of GTP-γ-S, the data displayed as open squares should rise to meet the data points displayed as filled circles. In only one cell of seven was there a small increase in the current measured without a prepulse, after addition of PMA. We measured the integral of charge movement with (300 μM) GTP-γ-S in the pipette before PMA that averaged 10.9 ± 1.5 pC, and 6 min after the addition of PMA it measured 10 ± 1.3 pC (not significant); a time by which PMA has always had its effect. The facilitation of the current in GTP-γ-S after a prepulse, at the same time points, was also measured. This was done by expressing the absolute amount of current facilitated as a percentage of the control current in GTP-γ-S.

FIG. 2. Long applications of 5-HT produce no desensitization, but application of PMA reverses the effect of 5-HT. A: graph of peak current against time. There is no desensitization evident in the inhibitory effect of 5-HT on Ca^{2+} current when 5-HT (1 μM) was applied for long periods of time. B: PMA can gradually counteract the effect of 5-HT in the continued presence of 5-HT when both are applied together at 1 μM. When the effect of 5-HT was washed off, 5-HT was less effective at inhibiting the Ca^{2+} current.
FIG. 3. Positive voltage does not allow PMA to reverse the effect of GTP-γ-S, and the rate of reinhibition of Ca$^{2+}$ current by GTP-γ-S after a facilitating prepulse is unchanged after PMA is added. A: in the control current traces (left), GTP-γ-S in the patch pipette produced an inhibition of dorsal raphe neuron Ca$^{2+}$ current that was reversed by long (200 ms) steps in the holding voltage of the cell to +80 mV, applied every 5 s. The same voltage pulse protocol was applied (right) in the presence of PMA (1 μM). This protocol did not allow PMA to reverse the effect of GTP-γ-S. B: integral of the charge carried by the inward movement of Ba$^{2+}$ ions, during the step to −20 mV, is shown plotted against time (open squares). Filled circles: integral of charge movement during the step to 0 mV, following the step to −80 mV. The figures in A, left occurred at time 1 indicated on the graph, and the figure in A, right occurred at time 2. PMA showed no trend to reverse the effect of GTP-γ-S. C: inhibition produced by GTP-γ-S (30 μM) in the pipette, in the absence, and its relief following a prepulse to +80 mV. Ba$^{2+}$ current traces were elicited by test pulses to 0 mV from a holding potential of −50 mV. The 30-ms prepulse to +80 mV was followed by a return to the holding potential for 10 ms to allow the Ba$^{2+}$ tail current to decay. The time between the prepulse and the test pulse was progressively increased from 10 to 200 ms. The traces have not been leak subtracted. No measure was made at time 0; but to facilitate the fitting of the data in this and all subsequent figures, the 1st measurement made at 10 ms was arbitrarily called time 0 and 100%, and 10 ms was deducted from each absolute X-axis value. D: average time course of the rate of reblock of the Ba$^{2+}$ current by GTP-γ-S. The experiment was conducted as shown in C. Facilitation of Ba$^{2+}$ current is expressed as a percentage of the peak current recovered after an interpulse interval of 10 ms. Reblock proceeded with a time course that was fit by a single exponential. The function used was \(f = Ae^{-\frac{t}{\tau}}\), where for the data with GTP-γ-S alone (●), \(A = 91.9\), \(\tau = 58.1\) (n = 5 and 6 with PMA), \(A = \) maximum current % (\(I_{\text{max}}\)), and \(x = \) time. The data were fitted with a simplex algorithm (Sigma plot). Also plotted on the graph are currents elicited as in C but in the presence of GTP-γ-S and PMA (1 μM; ■). In D some of the SE bars were smaller than the symbols.

of the action of the prepulse was measured by inserting a gap between the prepulse and the test pulse (Fig. 3C) (Elmslie and Jones 1990; Ikeda 1991; Penington et al. 1991). After the step to +80 mV and following the decay of the tail current, the action of GTP-γ-S (30 μM) was reversed by the prepulse. As the interval between the test pulse and the prepulse was varied between 10 and 200 ms, the effect of GTP-γ-S returned. Figure 3D plots the percentage of relief from GTP-γ-S inhibition as a function of the interval between prepulse and test pulse. The data were fitted by a single exponential; in six experiments the time constant (\(\tau\)) of the averaged data was 58.1 ms, similar to the rate of reinhibition exhibited by 1 μM 5-HT (56.8 ms) (Chen and Penington 1996). The rate of reinhibition is concentration dependent, being slower for lower concentrations of 5-HT, and is thought to be related to the amount of activated G protein free to interact with the Ca$^{2+}$ channel (Chen and Penington 1996; Elmslie and Jones 1994). When PMA (1 μM) was added, the size and shape of the currents in Fig. 3C did not change, and the plot of percentage relief from GTP-γ-S inhibition as a function of the interval between prepulse and test pulse...
When PMA is allowed to act before GTP-γ-S is perfused into the cell, the effect of GTP-γ-S is reduced

Next we tested whether the order of application of the phorbol ester and GTP-γ-S was an important determinant of the ability of PMA to modify the action of GTP-γ-S. Twelve cells were pretreated with PMA (1 μM) added to the pre-voltage-clamp bathing solution and the cells were exposed to PMA for 5 min before the recording solution was added (see METHODS). After this, the cell was patch clamped with GTP-γ-S (30 μM) in the patch pipette. After going whole cell, the onset of the effect of GTP-γ-S was always quick, but after pretreatment with PMA it appeared to be slightly delayed. This effect was difficult to measure because of the varying time taken to patch each cell after the whole cell conformation was achieved. With the use of a step to +70 mV, followed by a gap of 10 ms, we measured the degree of facilitation of the peak Ca2+ current at −20 mV immediately after establishing a whole cell recording, and at regular intervals thereafter. In control cells, the Ca2+ current with GTP-γ-S in the patch pipette was facilitated by 73.8 ± 7% (n = 8), but in the group of cells that had been pretreated with PMA the facilitation was only 33 ± 5% (n = 7, P < 0.001) (see Fig. 4). The size of the Ca2+ current in the control GTP-γ-S group, measured isochronally at the point at which the voltage-facilitated current peaks, was 823.2 ± 107 pA (n = 15), but in the group pretreated with PMA, the calcium current measured 1,259.6 ± 188 pA (n = 12); significantly bigger at the P < 0.05 level. This group contains some cells measured with a slightly different voltage protocol, so they could not be added to the measurements quoted above. The peak current after a facilitation pulse was not significantly different in the two groups. 5-HT (1 μM) applied after the GTP-γ-S effect had stabilized inhibited the control group calcium current by 7.6 ± 1.2% and the PMA-treated group by 5.8 ± 2% (not significant), suggesting that GTP-γ-S had reached its maximum effect in both conditions. The inhibition of the effect of GTP-γ-S by PMA is slightly bigger than the average 42% inhibition of the effect of 5-HT by PMA (Chen and Penington 1996). Thus we can account for all of the effect of PMA on the action of GTP-γ-S without invoking an additional effect of PMA on the receptor.

We also measured the rate of reinhibition of the Ca2+ current after a prepulse with GTP-γ-S in the pipette in cells that had been pretreated with PMA. In these cells the rate of reinhibition was slower, with a τ of the averaged data of 82 ms (n = 6) compared with the control value of 58.1 ms (Fig. 5). When the decay of prepulse facilitation from each cell was fitted with a single exponential, and these values were averaged, the τ after PMA pretreatment was 83.7 ± 11 ms, and for the cells where GTP-γ-S was added first, the τ was 60.4 ± 7.9. These values were significantly different (P < 0.05).

**Discussion**

The results of this study have revealed that when the G protein responsible for Ca2+ current modulation in DR neurons is directly stimulated with a nonhydrolyzable nucleotide analogue, thus bypassing receptor activation, the effect of GTP-γ-S is not reversed by PMA. This finding is different from the observations made in some other neuronal preparations (Swartz 1993; Zhu and Ikeda 1994). In the former study the effect of GTP-γ-S was reversed by PMA, occurring more readily after 200-ms steps to +80 mV every 5 s. The depolarization protocol may function to pull the GTP-γ-S-bound G protein away from the calcium channel, in this case allowing PKC access to its target either on the site on
the Ca\(^{2+}\) channel or the G protein at which they interact. Our findings are more readily explained by the proposal that although PKC can phosphorylate N-type channels (Ahlijanian et al. 1991), PKC does not exert its functional phosphorylation at the level of the Ca\(^{2+}\) channels on DR neurons or indeed on either site required for the binding interaction of the G protein and the Ca\(^{2+}\) channel. If PKC did effect a critical phosphorylation of either of these sites that can interfere with the action of GTP-\(\gamma\)-S, it should do so during the time that the effect of GTP-\(\gamma\)-S is reversed by depolarization to +80 mV. Even though we could reverse the effect of GTP-\(\gamma\)-S with positive voltage, PMA was ineffective at uncoupling the action of the G protein subunit, when receptor stimulation was bypassed. On the other hand, if in DR neurons PKC phosphorylates the G protein at the site at which GTP-\(\gamma\)-S would normally bind, or if PKC cannot phosphorylate the active conformation of the G protein (Sagi-Eisenberg 1989; Zick et al. 1986), this could explain a failure of PMA to reverse the effect of GTP-\(\gamma\)-S. This proposal was tested in the experiment in which the critical phosphorylation induced by PMA was allowed to occur before GTP-\(\gamma\)-S was introduced into the cell.

We have previously found that the rate of reinhibition of the Ca\(^{2+}\) current by 5-HT after a prepulse to +80 mV is concentration dependent (Chen and Penington 1996); this confirmed the findings of Elmslie and Jones (1994). This process may correspond to the rate of reassociation of the G protein subunit (perhaps \(\beta\gamma\)) with the calcium channel after it has been displaced by positive voltage (Herlitze et al. 1996; Ikeda 1996). When the G protein is activated by a receptor agonist, we also found that PMA decreased this rate of reblock. This finding can be interpreted as follows: perhaps fewer activated G proteins are available to interact with the Ca\(^{2+}\) channel after treatment with PMA. Another interpretation is also possible: phosphorylation of the Ca\(^{2+}\) channel may decrease the affinity of the activated G protein for the Ca\(^{2+}\) channel. However, the finding that PMA does not slow the rate of reblock or reverse the effect of GTP-\(\gamma\)-S, when GTP-\(\gamma\)-S is added first and temporally reversed with depolarization, appears to rule out this possibility. It was expected that the rate of reinhibition by intracellular GTP-\(\gamma\)-S should be similar to that with a maximal concentration of 5-HT, and because PMA does not reverse the effect of GTP-\(\gamma\)-S in these cells, the rate of reinhibition was expected to be unchanged on addition of PMA. This was confirmed in our study.

When PMA was applied before GTP-\(\gamma\)-S it was able to interfere with the action of subsequently perfused GTP-\(\gamma\)-S but could have no effect once GTP-\(\gamma\)-S was bound. This suggests that the critical phosphorylation effected by PKC is able to occur unhindered in the absence of GTP-\(\gamma\)-S but that the prior presence of GTP-\(\gamma\)-S is enough to totally prevent the PKC-dependent phosphorylation. PMA can reverse the activation of the G protein by the continual presence of 5-HT because 5-HT is thought to promote the GTP-bound (active) form of the G protein; this form is in equilibrium with the GDP-bound form, and presumably this species is vulnerable to phosphorylation by PKC in a way in which the irreversibly bound GTP-\(\gamma\)-S form is not. This finding suggests that in some types of neurons PMA does phosphorylate the Ca\(^{2+}\) channel or G protein (preventing their interaction) when the G protein-bound-GTP-\(\gamma\)-S dissociates, but in others, the critical response-related effect is exerted only on the G protein. We recognize that the action of PMA in
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intact cells may be subtly different from its effect when it is applied to a whole cell clamped neuron. However, any possible difference in the effects of PMA cannot explain differing results between studies that find PMA can reverse the effect of GTP-γ-S and those that do not, because in all studies PMA is applied during whole cell recording. The simplest explanation is that order of application is important for the action of PMA in DR neurons but further clarification of this point may be informative.

We also studied the rate of association of the G protein subunit responsible for GTP-γ-S-induced inhibition of Ba2+ current, after a prepulse to +80 mV, before or after pretreatment with PMA. We predicted that as pretreatment with PMA was shown to partially prevent the effect of GTP-γ-S, the rate of reactivation of the Ca2+ current by GTP-γ-S after a prepulse should also be slowed. This was also found to be the case in this study.

In cells in which the effect of GTP-γ-S can be reversed by PMA, Swartz suggested that the site phosphorylated by PKC may be masked by a bound activated G protein, and PKC might only work when the G protein was dissociated from the Ca2+ channel; thus PMA could act on either constituent at the site at which the Ca2+ channel or the G protein interact. Our data indicate that in DR neurons the site of phosphorylation by PKC is actually on the G protein itself (Zick et al. 1986), being the site at which GTP-γ-S interacts, and perhaps only on a specific GDP-bound conformation. In this respect our data in serotonergic neurons differ from the situation in cortical and some other neurons.

Possible reasons for the differences between the results of Swartz and Zhu and Ikeda on the one hand, and Golard et al. (1993) between our results and those of Golard et al. (1993) is that the one exception is that we used Tris methanesulphonate as the main salt instead of TEACl. We found that our currents were very small when TEACl was used. Other than the ability of PMA to reverse the effect of GTP-γ-S, most aspects of our findings were similar. PMA was shown to partially prevent the effect of GTP-γ-S, most aspects of our findings were similar.