Patch Cramming Reveals the Mechanism of Long-Term Suppression of Cyclic Nucleotides in Intact Neurons

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Cyclic nucleotides (cAMP and cGMP) play a central role in synaptic plasticity. The nitric oxide (NO)/cGMP pathway may be crucial for long-term depression (LTD) in cerebellar Purkinje neurons (Lev-Ram et al., 1997), and both NO/cGMP and cAMP have been implicated in long-term potentiation (LTP) in the hippocampus (Arancio et al., 1995; Nicol and Malenka, 1995). Despite the growing awareness of the involvement of cyclic nucleotides in synaptic plasticity, relatively little is known about the intracellular dynamics of cAMP or cGMP.

We recently described a method for real-time detection of free cGMP in single living cells by “patch cramming,” in which CNG channels engineered to be especially sensitive and selective for cGMP are used as biosensors (Trivedi and Kramer, 1998). A patch pipette is used to excise a membrane patch from a Xenopus oocyte expressing a high density of cGMP detector channels. The detector patch is calibrated with known concentrations of cGMP and then “crammed” into a recipient N1E-115 neuroblastoma cell. The activity of the channels provides a continuous quantitative measure of cGMP concentration in the recipient cell.

N1E-115 neuroblastoma cells are related biochemically and physiologically to sympathetic neurons (Kimhi et al., 1976). We found that NO donors or muscarinic agonists elevate cGMP in both cells (Trivedi and Kramer, 1998). There was no decrement of the cGMP response generated by NO, whereas cGMP transients diminished dramatically with repeated activation of muscarinic receptors. Surprisingly, muscarinic activation also suppressed subsequent cGMP responses to NO for at least 30 min. Biochemical measurements confirmed that this long-term suppression (LTS) of cGMP occurs in both cell types and persists for up to 2 hr. Thus muscarinic activation not only elevates cGMP in the short term, it also depresses resting cGMP and prevents NO-elicited transients in the long term.

The biochemical steps linking muscarinic receptors to cGMP elevation have been studied extensively and are conserved in sympathetic neurons and neuroblastoma cells. Activation of muscarinic receptors leads to G-protein-mediated inositol triphosphate (IP3) production and subsequent Ca2+ release from intracellular stores (Briggs et al., 1985; Thompson et al., 1995). Elevated cytoplasmic Ca2+ activates NO synthase (NOS) to produce NO (Forstermann et al., 1990; Wotta et al., 1998), which then stimulates cGMP production by soluble guanylate cyclase (sGC) (Hu and El-Fakahany, 1993). The purpose of this study is to determine the mechanism of LTS. Because NO directly activates sGC, the suppression of cGMP responses elicited by NO donors must result from the regulation of enzymes involved in cGMP metabolism, far downstream from the muscarinic receptor itself. These include sGC, which synthesizes cGMP, and phosphodiesterase (PDE), which degrades cGMP. How do muscarinic agonists initiate LTS? Because cGMP itself does not produce LTS, some upstream intermediate in the muscarinic signaling cascade must feed forward to regulate sGC and/or PDE. How is LTS maintained for hours...
after brief (1 min) muscarinic stimulation? The long duration of LTS suggests that the regulation of these enzymes is mediated by some covalent modification, such as phosphorylation. Via patch clamp measurements, pharmacological treatments, and direct injection of cGMP and enzyme inhibitors, we answer these questions and elucidate the mechanism of LTS.

**MATERIALS AND METHODS**

**Cell culture.** N1E-115 neuroblastoma cells (Amano et al., 1972) were maintained in DMEM with 10% FBS at 37°C. The neurons were allowed to mature for 8–10 d. After 2–7 d the vitelline membrane was removed, and the denuded oocytes were placed in the experimental chamber with neuroblastoma cells, which were used as recipient cells in all patch-clamping experiments. Dechorionated oocytes (2–4 MN) were filled with a solution containing (in mM): 100 K-gluconate, 20 KCl, and 10 Na-HEPES, pH 7.4. This also served as the cGMP calibration solution. After the formation of a gigaohm seal the inside-out patches were excised, and the patch pipette was placed in the outlet of a 1 mm in diameter tube for the application of four to five concentrations of cGMP (0.5–100 μM). Current responses through CNG channels were obtained with a Warner PC-505 patch clamp, filtered at 1 kHz, digitized, stored, and later analyzed on a computer. To confirm successful impalement of recipient neuroblastoma cells and to allow voltage clamp, we used a second pipette containing nystatin for perforated patch clamp (Horn and Marty, 1988; Trivedi and Kramer, 1998). When cell resistance declined to <60 MΩ, the membrane potential was held under voltage clamp (−50 mV), and the neuroblastoma cell was impaled with the detector patch electrode. Successful impalement was confirmed by the appearance of an offset in the oocyte patch current caused by the potential difference across the neuroblastoma cell membrane. During the calibration the oocyte patch was held at −75 mV, and 350 μA negative current voltage pulses to 0 mV were applied at 2 Hz. Resulting currents were measured to calculate conductance changes in response to cGMP. After insertion into the recipient cell the detector patch was placed at −25 mV so that the total voltage difference across the detector patch was the same as during the calibration (−75 mV). Changes in intracellular cGMP were recorded as changes in detector patch conductance.

**Solutions** were superfused continuously over the neuroblastoma cell. The bath solution contained (in mM): 120 NaCl, 2.5 KCl, 1 MgCl2, 2 CaCl2, 5 Na-HEPES, and 10 d-glucose, pH 7.4. All pharmacological agents including cGMP channels were obtained with a Warner PC-505 patch clamp, filtered at 1 kHz, digitized, stored, and later analyzed on a computer. To confirm successful impalement of recipient neuroblastoma cells and to allow voltage clamp, we used a second pipette containing nystatin for perforated patch clamp (Horn and Marty, 1988; Trivedi and Kramer, 1998). When cell resistance declined to <60 MΩ, the membrane potential was held under voltage clamp (−50 mV), and the neuroblastoma cell was impaled with the detector patch electrode. Successful impalement was confirmed by the appearance of an offset in the oocyte patch current caused by the potential difference across the neuroblastoma cell membrane. During the calibration the oocyte patch was held at −75 mV, and 350 μA negative current voltage pulses to 0 mV were applied at 2 Hz. Resulting currents were measured to calculate conductance changes in response to cGMP. After insertion into the recipient cell the detector patch was placed at −25 mV so that the total voltage difference across the detector patch was the same as during the calibration (−75 mV). Changes in intracellular cGMP were recorded as changes in detector patch conductance.

**RESULTS**

**Which metabolic enzyme underlies LTS?** We began by investigating the possibility that stimulation with a muscarinic agonist (oxo-M) induces LTS by generating some molecule that “scavenges” NO and prevents it from activating sGC, thereby suppressing cGMP production. However, we find that LTS applies not only to cGMP transients elicited by NO donors but also to responses elicited by YC-1, a specific sGC activator that acts in an NO-independent manner (Wu et al., 1995) (Fig. 1A). Application of YC-1 without the previous induction of LTS effectively elevates cGMP (Fig. 1B). Hence LTS is not specific to NO, discounting the possibility that the generation of an NO scavenger is the mechanism.

Does a decrease in sGC activity underlie LTS? Previously, we showed that isobutyl methylxanthine (IBMX), a PDE inhibitor (Beavo et al., 1982), elicits a rise in cGMP (Trivedi and Kramer, 1998), suggesting that in the absence of degradation there is significant basal sGC activity. We also showed that responses to saturating IBMX (100 μM) are not reduced after LTS induction, indicating that basal guanylate cyclase activity is not inhibited. Is the guanylate cyclase enzyme responsible for basal cGMP production the same as the enzyme that underlies NO-elicited GMP production? cGMP transients in response to IBMX are blocked by a sGC inhibitor (Garthwaite et al., 1995), ODQ (1H-[1,2,4]oxadiazolo [4,3-a]quinazolin-1-one; n = 4), or a NOS inhibitor (Silva et al., 1995), 7-nitro indazole (7-NI; n = 3), both of which prevent cGMP transients in response to oxo-M (Trivedi and Kramer, 1998). Hence both sGC and NOS are required not only for muscarinic responses but also for basal cGMP production. Taken together, these results are inconsistent with inhibition of sGC during LTS and focus our attention on PDE.

To test directly whether PDE activity is increased during LTS, we bypassed sGC by pressure-injecting cGMP into recipient cells while measuring intracellular cGMP with the detector patch. The cGMP concentration in the injection pipette and the duration of pressure injection were adjusted to produce peak intracellular cGMP elevations of <5 μM. Before LTS the injection of cGMP rapidly (~1 sec) activated the detector patch, and the cGMP elevation decayed very slowly over 10 min (Fig. 2A; n = 6), suggesting the slow degradation of cGMP. After LTS induction the cGMP injection produced smaller responses that decayed
much more rapidly (<1 min; n = 9). To confirm that enhanced PDE activity is responsible for the dramatically accelerated decay, we injected cGMP in the presence of IBMX after inducing LTS (Fig. 2B). With IBMX present the cGMP injections resulted in large, long-lasting responses indistinguishable from those elicited before LTS induction. In this experiment 10 μM ODQ was included to block all sGC activity irreversibly and prevent IBMX itself from elevating cGMP. These results conclusively demonstrate that the enhancement of PDE activity underlies LTS.

Some types of PDE are specific for cGMP or cAMP, but others have broad substrate specificities and can hydrolyze either cyclic nucleotide (Beavo, 1995). To test whether the PDE(s) upregulated during LTS also can hydrolyze cAMP, we used an enzyme immunoassay that is specific for cAMP. Figure 3A shows that elevations of cAMP in cultures of mamalian sympathetic neurons, elicited by vasoactive intestinal peptide (VIP), are suppressed after oxo-M treatment. As a control, repeated applications of VIP elicited reproducible elevations of cAMP (Fig. 3B). Suppression of cAMP after muscarinic activation also was observed in similar experiments in neuroblastoma cells, and the suppression persisted for at least 30 min (data not shown). Thus LTS applies both to cGMP and cAMP, further supporting the conclusion that the enhanced activity of one or more PDEs is responsible.

To investigate which PDEs are important for regulating cGMP levels and LTS, we applied various selective inhibitors at concentrations that fully block specific families of PDEs. Neither zaprinast (10 μM), selective for cGMP-specific PDEs (PDE-5) (Gillespie and Beavo, 1989), nor RO-20-1724 (100 μM), selective for cAMP-specific PDEs (PDE-4) (Tanner et al., 1986), elevated basal cGMP (n = 3), suggesting that the PDEs involved in LTS are not PDE-4 or PDE-5. Likewise, neither 100 μM vinpocetine (Ahn et al., 1989) nor 10 μM EHNA (Mery et al., 1995), selective for Ca²⁺/calmodulin-dependent PDEs (PDE-1) and cGMP-stimulated PDE (PDE-2), respectively, elevated cGMP (n = 3). Quazinone (20 μM), an inhibitor of cGMP-inhibited PDEs (PDE-3) (Holck et al., 1984), did elevate cGMP to the same extent as did saturating IBMX (100 μM; n = 3), a nonselective PDE inhibitor. Hence the upregulation of PDE-3 may underlie LTS, but it is also possible that pharmacologically uncharacterized PDEs in other families or even a novel form of PDE is responsible.

How is LTS initiated?

Muscarinic agonists transiently elevate cGMP and subsequently cause LTS. Our results suggest that some intermediate in the muscarinic signaling cascade feeds forward to enhance PDE activity. Similarly, several other transmitters (angiotensin, bradykinin, and neurotensin) initially elevate cGMP but subsequently induce LTS (n = 2–3 for each transmitter). These transmitters may act via a common signaling cascade involving the G-protein-mediated production of IP₃, Ca²⁺ release from internal stores, Ca²⁺ activation of NOS, production of NO,
and activation of sGC. Because prolonged application of NO alone does not cause LTS (Trivedi and Kramer, 1998), the signal responsible for inducing LTS must be upstream of NO. Because Ca\(^{2+}\) is an essential component of signaling for each of these transmitters, we asked whether it is crucial for inducing LTS.

To test whether a rise in intracellular Ca\(^{2+}\) is sufficient for initiating LTS, we used the Ca\(^{2+}\) ionophore ionomycin. Figure 4A shows that ionomycin, which raises cytoplasmic Ca\(^{2+}\) even in the absence of external Ca\(^{2+}\) (Albert and Tashjian, 1986; our unpublished observations), produces a rise in cGMP and induces LTS (n = 3). Depletion of intracellular Ca\(^{2+}\) stores by pretreatment with thapsigargin prevented the rise in cGMP in response to oxo-M and prevented the induction of LTS (n = 3; Fig. 4B). These findings suggest that Ca\(^{2+}\) mobilization is both necessary and sufficient for induction of LTS.

To test further the role of Ca\(^{2+}\) in LTS, we pressure-injected BAPTA into the recipient neuroblastoma cell to chelate intracellular Ca\(^{2+}\). When BAPTA was injected before the application of oxo-M, the muscarinic agonist was unable to elevate cGMP, and subsequent NO responses were not suppressed (Fig. 5A), strongly supporting the idea that Ca\(^{2+}\) is necessary for induction. A different result was obtained when BAPTA was injected after LTS was initiated (Fig. 5B). Once LTS is triggered, BAPTA is unable to reverse the suppression of cGMP and restore responses to NO or muscarinic agonists, suggesting that LTS can be maintained even in the absence of intracellular Ca\(^{2+}\). Moreover, the time course of LTS (up to 2 hr) is much longer that the duration of the Ca\(^{2+}\) transient resulting from muscarinic activation, which in Ca\(^{2+}\) imaging experiments appears to be <60 sec (n = 3; data not shown), in agreement with previous studies (Mathes and Thompson, 1994). Hence the maintenance of LTS is Ca\(^{2+}\)-independent.
induction. However, the injection of "CaM-KIIotide," a highly potent and selective peptide inhibitor of CaM-KII (Chang et al., 1998), not only prevented induction when injected before oxo-M application (data not shown) but, when injected after oxo-M application, resulted in the reversal of LTS, restoring the NO-elicited rise in cGMP to $89 \pm 7\%$ ($n = 3$) of the response (Fig. 7D). Hence maintained activity of CaM-KII appears to be necessary for the maintenance of LTS.

**DISCUSSION**

**The mechanism of LTS**

Activation of muscarinic receptors in neuroblastoma and sympathetic neurons elevates cGMP in the short term but delayed and over a prolonged time course also suppresses the elevation of both cGMP and cAMP. The net effect is a single brief rise followed by
a nearly complete shutdown of cyclic nucleotide signaling that persists for hours. Figure 8 illustrates our proposed mechanism for the elevation and subsequent suppression of cGMP. Muscarinic receptors (M₁ subtype) act via a G-protein (Gq) to activate PLC, which produces IP₃. IP₃ triggers Ca²⁺ release from storage organelles, increasing the concentration of intracellular Ca²⁺. Ca²⁺ mobilization and the resulting increase in cytoplasmic Ca²⁺ are necessary and sufficient for triggering both the initial cGMP signal and, with a delay, the subsequent LTS. The rise in cGMP occurs within 10 sec after oxo-M stimulation (Trivedi and Kramer, 1998), whereas the fall in cGMP once LTS is initiated requires >30 sec (see Fig. 2B), consistent with LTS involving additional biochemical steps.

The Ca²⁺-elicited increase in cGMP involves Ca²⁺/calmodulin-dependent NO activation, production of NO, and stimulation of sGC (Christopoulos and El-Fakahany, 1998; Trivedi and Kramer, 1998). The crucial Ca²⁺ effector involved in the longer-lasting phenomenon of LTS appears to be CaMKII. Three selective inhibitors of CaMKII (KN-62, AIP, and CaM-KIINtide) all prevent LTS induction. Induction also is prevented by BAPTA injection, consistent with a Ca²⁺-dependent triggering mechanism. However, once initiated, BAPTA is ineffective at reversing LTS. The observation that Ca²⁺ is required for inducing, but not maintaining, LTS coincides with the known behavior of CaMKII, which loses its Ca²⁺ sensitivity with autophosphorylation. The distinction between induction and maintenance is strikingly similar to the LTP of synaptic transmission in the hippocampus, which also involves CaMKII autophosphorylation (Malinow et al., 1988; Barria et al., 1997; Giese et al., 1998).

LTS maintenance could result from persistent CaMKII activity, leading to continual phosphorylation of PDE or other relevant substrates. In contrast, LTS might be maintained if the substrate remained stably phosphorylated, even after CaMKII activity declines. CaMKII peptide inhibitor experiments help to distinguish between these possibilities. Although AIP prevents induction, it only partly reverses the maintenance of LTS, similar to the effects of this peptide on hippocampal LTP (Otmakhov et al., 1997). However, CaM-KIINtide, a higher-affinity inhibitor, possibly more stable to intracellular degradation, does eliminate the maintenance of LTS. The observation that the maintenance of LTS can be "erased" by the injection of CaM-KIINtide suggests that CaMKII must be persistently active for LTS to endure.

Our results indicate that the enzyme that underlies LTS is PDE and not sGC. CaMKII-dependent phosphorylation of PDE, or a protein that regulates PDE activity, increases enzyme activity, severely blunting cGMP responses and reducing the resting level of cGMP. It will be interesting to identify the specific PDE(s) responsible for LTS and to determine biochemically whether they are phosphorylated directly by CaMKII. PDEs found in neuroblastoma (Giorgi et al., 1993) and sympathetic neurons (Capuzzo et al., 1986) include isoforms that are cAMP-specific (PDE-4), cGMP-specific (PDE-5), and nonspecific (PDE-1, -2, and -3) for cAMP and cGMP. Theoretically, LTS could result from augmentation of either a nonspecific PDE alone or a combination of two or more of the cyclic nucleotide-specific forms.

PDE-1 was the first enzyme shown to be activated by Ca²⁺ via the Ca²⁺ binding protein calmodulin (Cheung, 1980). Although there is strong precedent for PDE activation by increased intracellular Ca²⁺, this classical regulatory mechanism probably is not involved in the longer-lasting phenomenon of LTS. LTS appears to be mediated by a covalent modification of PDE, namely phosphorylation, rather than allosteric regulation by Ca²⁺/calmodulin, which would disappear rapidly when Ca²⁺ levels decline. CaMKII phosphorylation has been shown to inhibit rather than enhance PDE-1 activity (Beavo, 1995), and our finding that vinpocetine, a selective inhibitor of this enzyme, does not elevate cGMP suggests that PDE-1 is not involved in LTS. Likewise, results with other selective PDE inhibitors suggest that PDE-2, -4, and -5 are not involved, leaving PDE-3, pharmacologically uncharacterized PDEs in other PDE families, or perhaps a novel PDE as the responsible enzyme.

Neurotransmitters that mobilize Ca²⁺, including angiotensin, bradykinin, neurotransin, and acetylcholine, can trigger LTS. However, moderate depolarization to open voltage-gated Ca²⁺ channels (trains of 1 sec pulses to 0 mV) is ineffective in elevating cGMP or inducing LTS (our unpublished results). It is possible that there is insufficient Ca²⁺ influx with depolarization to trigger cGMP production and LTS induction. Furthermore, the location of the Ca²⁺ transient induced by mobilization, but not by depolarization, may be optimal for activating the NOs and CaMKII. Perhaps in these cells NOs and CaMKII are localized near sites of Ca²⁺ release (e.g., the ER) rather than near Ca²⁺ channels in the plasma membrane.

Functional implications of LTS

The biochemical machinery involved in cGMP production and LTS induction and maintenance is common to many cell types. The ubiquity of these proteins suggests that LTS may occur in other cells in which signals that trigger Ca²⁺ mobilization occur simultaneously with signals that use cGMP or cAMP, possibly providing a mechanism for long-term plasticity of transmitter interactions. It will be interesting to determine whether LTS occurs in cerebellar Purkinje neurons, in which both IP₃-mediated Ca²⁺ release (Finch and Augustine, 1998) and NO-induced cGMP production (Lev-Ram et al., 1997) have been implicated in the long-term depression of synaptic transmission. Sympathetic neurons exhibit a form of LTP (Briggs and McAfee, 1988), and it is possible that LTS plays an important role in this process. Because cGMP and/or cAMP in sympathetic ganglia are involved in synaptic modulation (Briggs, 1992; Wu and Dun, 1996), regulation of neurotransmitter metabolism (Ip et al., 1985),
and growth factor-mediated survival (Farinelli et al., 1996), the shutdown of cyclic nucleotide signaling also may have other important physiological consequences.

Cyclic nucleotides affect cell function via two main classes of effectors: CNG channels and protein kinases (PKA and PKG), which have strikingly different sensitivities to cyclic nucleotides. CNG channels are activated half-maximally by cGMP and cAMP at 2–100 μM (Zagotta and Siegelbaum, 1996), whereas PKA and PKG have apparent Kₜ₅ values that range from 4 to 150 nM (Shabb et al., 1990). Our cGMP detection method is based on a highly sensitive CNG channel (Kᵢ₅ = 4 μM), but 0.5 μM is the lower limit of detection. Transmitters such as acetylcholine and NO could induce undetectable changes in cGMP concentration possibly important for regulating PKA and PKG but that are outside the range that would be important for regulating CNG channels. In fact, LTS may provide a mechanism for toggling the primary effectors of cyclic nucleotide signaling from channels to kinases by resetting the basal concentration and operating range of cAMP and cGMP.

There are many possible “nodes” of interaction enabling cross talk between Ca²⁺ and cyclic nucleotide signaling systems in cells. Ca²⁺, acting via calmodulin, can activate or inhibit adenylate cyclase and also activate PDE-1. More indirectly, Ca²⁺ affects the activity of several protein kinases and phosphatases, which can modulate receptors, G-proteins, adenylate cyclase, various PDEs, and protein kinases. By monitoring cGMP directly, we have succeeded in identifying the specific node in the signaling circuitry (CaMKII-dependent regulation of PDE) that predominates in the regulation of cyclic nucleotides in neuroblastoma cells and sympathetic neurons.

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