**Ca^{2+}/calmodulin-dependent kinase II mediates simultaneous enhancement of gap-junctional conductance and glutamatergic transmission**

(abstract)

While chemical synapses are very plastic and modifiable by defined activity patterns, gap junctions, which mediate electrical transmission, have been classically perceived as passive intercellular channels. Excitatory transmission between auditory afferents and the goldfish Mauthner cell is mediated by coexisting gap junctions and glutamatergic synapses. Although an increased intracellular Ca^{2+} concentration is expected to reduce gap junctional conductance, both components of the synaptic response were instead enhanced by postsynaptic increases in Ca^{2+} concentration, produced by patterned synaptic activity or intradendritic Ca^{2+} injections. The synchronously induced potentiations were blocked by intradendritic injection of KN-93, a Ca^{2+}/calmodulin-dependent kinase (CaM-K) inhibitor, or CaM-KIINtide, a potent and specific peptide inhibitor of CaM-KII, whereas the responses were potentiated by injection of an activated form of CaM-KII. The striking similarities of the mechanisms reported here with those proposed for long-term potentiation of mammalian glutamatergic synapses suggest that gap junctions are also similarly regulated and indicate a primary role for CaM-KII in shaping and regulating interneuronal communication, regardless of its modality.

The degree of interneuronal communication via chemical synapses is a dynamic feature of the central nervous system and is mainly determined by the synapses’ own activity (1). In contrast, gap junction-mediated electrical synapses (2) are considered to be largely unmodifiable. Recent studies indicate, however, that these synapses also can undergo activity-dependent potentiation. As with chemical synapses, patterned synaptic activity has been shown to produce short- and long-term modifications of interneuronal coupling (3, 4).

Eighth nerve afferents (see Fig. 1a) terminate on the lateral dendrite of the M-cell as individual “large myelinated club endings” (5). Stimulation of the posterior branch of the eighth nerve evokes a mixed excitatory synaptic response in the dendrite (Fig. 1b) composed of a fast electrical potential followed by a chemical EPSP mediated by glutamate (6, 7). Because of the fast membrane time constant of the M-cell (about 400 ms; ref. 8), these two kinetically distinct components can be easily distinguished and reliably measured (Fig. 1b), providing a unique opportunity to dynamically explore modifications of junctional conductance under different physiological conditions.

As shown previously (3, 4), discontinuous high-frequency stimulation of the eighth nerve quickly induces homosynaptic potentiation (Fig. 1c) of both the electrical and glutamatergic components (3), which usually persist for the remainder of the experiment (up to 2 hr). However, the changes observed in both components of the synaptic response can be transient (4), lasting only for a period of 3–10 min, suggesting in the case of these gap junctions that junctional conductance is tightly regulated, perhaps involving post-translational modifications of gap junctional channels. These short-term poteniations (STP; ref. 4) are likely to constitute the early phase of the long-lasting poteniations (LTP; ref. 3), since in both cases the induction depends on the activation of N-methyl-D-aspartate (NMDA) receptors and an increase in postsynaptic intracellular Ca^{2+}. That is, they can be prevented by NMDA receptor antagonists and previous intradendritic injections of the Ca^{2+} chelator, 1,2-bis(2-aminophenoxy)ethane-N,N',N',N''-tetraacetaete (BAPTA) (3, 4). The apparent role of Ca^{2+} in regulating the intracellular concentration of Ca^{2+} should rather suppress gap junction conductance, given the direct inhibitory action of this cation on gap junctional conductance (9). On the other hand, stimulating paradigms known to promote an increase in presynaptic Ca^{2+} concentration (e.g., paired pulses, continuous high-frequency stimulation) were ineffective at inducing changes in junctional conductance (4).

We therefore investigated the role of Ca^{2+} and its molecular mechanism(s) in modulating both the electrical and chemical components of synaptic transmission in the goldfish M-cell. On the basis of this indirect evidence, we postulated that gap junctional conductance at these mixed synapses can be potentiated as a consequence of post- but not presynaptic elevations in Ca^{2+} concentration. The results presented here show that, in accord with this prediction, activity-dependent modulation of gap junctional conductance and glutamatergic transmission relies on a postsynaptic increase in the intracellular concentration of Ca^{2+}, which in turn leads to activation of Ca^{2+}/calmodulin-dependent kinase II (CaM-KII), an essential step in the induction of the modifications.

**MATERIAL AND METHODS**

**Experimental Arrangement.** Responses to stimulation of the posterior eighth nerve or to antidromic stimulation of the spinal cord were recorded intracellularly *in vivo* from the M-cell’s lateral dendrite. The intracellular electrode was also inserted into the posterior end of the Mauthner cell. The Methods section is available in the full paper.

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used for iontophoretic or pressure injections. Responses were quantified after averaging sets of 20 or more consecutive traces. Student’s t test was used to assess statistical significance. Errors are presented as 1 SEM. To obtain activity-dependent potentiation, discontinuous tetanic stimulation of the nerve was used (trains of 4–6 pulses at 500 Hz applied every 2 s during 4 min; strength was sufficient to orthodromically activate the M-cell at least once per train; see refs. 3 and 4). Intraterminal recordings of large myelinated club endings (5–15 μm in diameter) were obtained at about 20 μm lateral to the initial penetration of the M-cell’s lateral dendrite.

**Intracellular Injections.** For intradendritic injections the following compounds were added to the recording vehicle solutions and pressure injected into the M-cell’s lateral dendrite (see ref. 4): CaCl$_2$ (2–6 mM in 0.5–2.5 M KCl/10 mM Hepes, pH 7.2); EGTA (5 mM in 2.5 M KCl/10 mM Hepes, pH 7.2); or KN-93, a CaM-K inhibitor (Seikagaku America, Rockville, MD; 200–300 μM in 0.5 M KCl/10 mM Hepes, pH 7.2). CaM-KIIIn tide, a potent and specific peptide inhibitor of CaM-KII, was injected iontophoretically.

**Immunohistochemistry and Immunoblot Analysis.** Affinity-purified samples of anti-peptide CaM-KII antibodies directed against sequences in the autoregulatory domain of the rat brain α subunit (G-301) or at the COOH-terminus of the association domain of the β/β’ subunits (RU-16) were used in all experiments (11). Fish were perfused with 4% paraformaldehyde and brains were stored overnight. Vibratome sections (50 μm) were rinsed with PBS, incubated with blocking cocktail and centrifuged at 4°C, and the supernatant was collected. Approximately 4 μg of protein was used per assay, which took place in a standard mixture of 50 mM Hepes at pH 7.5 (20 mM for PKC), 1 mM DTT, 10 mM MgCl$_2$, and 0.4 mM [γ-32P]ATP. For CaM-KII, the activators were 0.8 mM Ca$^{2+}$ and 2 μM calmodulin; for PKA, the activator was 1 mM cAMP.
with 1 mM 3-isobutyl-1-methylxanthine; and for PKC it was 0.3 mM Ca²⁺, 0.14 mM phosphatidyserine, and 3.8 μM diacylglycerol. The substrates for CaM-KII, PKA, and PKC were 40 μM Syntide 2 (Peptide Express, Fort Collins, CO), 100 μM Kemptide (Peninsula Laboratories), and 100 μM epidermal growth factor receptor (EGFR) peptide, respectively.

RESULTS

Effects of Pre- and Postsynaptic Injections of Ca²⁺. On the basis of indirect evidence, we postulated that gap junctional conductance at these mixed synapses can be potentiated as a consequence of post- but not presynaptic elevations in Ca²⁺ concentration. We directly tested this hypothesis by performing intradendritic and intraterminal recordings and injections close to the synapses themselves, a unique experimental advantage of this system. As predicted, intradendritic pressure injections of Ca²⁺ enhanced both components of the synaptic response. These enhancements grew slowly over a period of 10–15 min, attaining postsynaptic response magnitudes of approximately 150% of controls (Fig. 1 d–f). These Ca²⁺-induced potentiations occluded those evoked by eighth nerve tetani (n = 3, not shown). Response amplitudes for the electrical and chemical postsynaptic potentials (PSPs) and antidromic spike measured after at least 20 min of recording without Ca²⁺ in the electrode solution were not significantly different from controls (Fig. 1 f). Thus, postsynaptic elevations of Ca²⁺ triggered an enhancement of gap junctional conductance.

Intraterminal Ca²⁺ injections (Fig. 2a) did not enhance gap junctional conductance as measured by the electrotonic coupling potential in the terminal due to the passive dendritic depolarization produced by the antidromically evoked M-cell action potential (Fig. 2b Upper). Rather, they actually decreased the amplitude of the coupling potentials, an effect associated with a hyperpolarization of the terminals (Fig. 2b). On average (n = 11), the terminal resting potential was hyperpolarized, from 64.6 ± 1.5 mV to 73.4 ± 2.7 mV, and the coupling potential amplitude was reduced from 4.9 ± 0.4 mV to 3.7 ± 0.4 mV (Fig. 2c). Iontophoretic Ca²⁺ injections of shorter duration (not shown) resulted in reversible changes in both parameters, suggesting that these effects may be due to the activation of a Ca²⁺-dependent potassium conductance present at many synaptic terminals (12). Moreover, the decrease in the amplitude of the electrotonic potential can also be attributed to its previously shown voltage dependence in the terminal (13). Consistent with this finding, intraaxonal Ca²⁺ injections (afferent recording site in the eighth nerve root), performed while simultaneously recording the lateral dendrite, did not modify the amplitude of the orthodromically evoked unitary coupling potential, which averaged 94.1% ± 3.6% of control (n = 5). Therefore, the Ca²⁺-dependent process that triggers the modifications in junctional conductance is most likely restricted to the presynaptic hemichannels.

Synaptically Induced Potentiations: Evidence for the Involvement of CaM-KII. To determine whether induction of the enhancements depends on a localized rather than generalized intradendritic increase of Ca²⁺, we compared (14) the blocking efficacy of a slower Ca²⁺ buffer, EGTA, with that previously shown for a faster one, BAPTA (4). EGTA (5 mM) injected intradendrically prior to tetanic stimulation was a significantly less effective blocker of the potentiations than was 5 mM BAPTA. In the presence of BAPTA, tetanic stimulation transiently depressed both components of the synaptic response, whereas a clear potentiation was observed with EGTA (Fig. 3a). To accurately measure the effect of these buffers or other compounds (see above) on the induction of these rapid activity-dependent potentiations, we compared the averages of the last 15–40 traces obtained before and after tetanic stimulation. The dramatic difference between the effects of EGTA and BAPTA (Fig. 3e) suggests that a localized increase in Ca²⁺ is indeed responsible for the induction process.

Because this localized increase seems to be synaptically mediated through activation of NMDA receptor channels, the Ca²⁺ sensor responsible for the induction should be localized in the postsynaptic cell close to these channels. A likely candidate was multifunctional CaM-KII, whose α-subunit represents a major protein in the postsynaptic densities (PSDs; ref. 15) of vertebrate synapses and has a primary role in modulating neuronal synaptic plasticity (16–18). To test for involvement of CaM-KII, we first injected KN-93, a CalM-K inhibitor. As shown in Fig. 3b, KN-93 injection blocked both components of

![Fig. 2. Presynaptic injections of Ca²⁺ do not increase junctional conductance. (a) Intraterminal recordings were obtained from large myelinated club endings, and Ca²⁺ was injected iontophoretically. (b) After Ca²⁺ injection the terminal was hyperpolarized from −62 to −72 mV in this case, and the antidromic coupling potential was decreased to approximately 70% of control (Upper). (Lower) Plot of the amplitude of the antidromic coupling potential versus time (each point represents the average of 10 traces) for the same experiment. (c) Diagram summarizing the values of the resting potential and coupling potential amplitudes (AD coupling) obtained for 11 terminals in control (●) and after at least 10 min of continuous Ca²⁺ injection ○. The changes were statistically significant.](image-url)
the synaptic response immediately after a tetanus, and this effect was reversible with time, presumably because of washout of the drug (Fig. 3b).

The recent cloning of a CaM-KII inhibitory protein (CaM-KIIN) and a related 28-residue peptide that contains the inhibitory potency and specificity (CaM-KIINtide; see ref. 19) provided an alternative means to examine the effect of selective inhibition of CaM-KII on the induction of activity-dependent potentiations. Although postsynaptic injection of other CaM-KII inhibitory peptides has been shown to block induction of long-lasting potentiation in region CA1 of mammalian hippocampus, those peptides have limited CaM-KII specificity and also inhibit several other kinases, thereby complicating interpretation of their effects (20–23).

In contrast, CaM-KIINtide was shown to be a potent and highly specific inhibitor of CaM-KII activity from several species (19). The effect of CaM-KIINtide on CaM-KII, PKA, and PKC activities in soluble extracts from goldfish brain homogenates was tested (Fig. 4d). CaM-KIINtide inhibits goldfish CaM-KII with an IC_{50} of 300–400 nM, and it showed no significant inhibition of PKA or PKC at concentrations up to 10 μM. In confirmation of our hypothesis, intradendritic injections of this peptide prevented the activity-dependent potentiations (Fig. 3c). For 12 experiments in which the effect of this peptide was tested, the electrical and chemical components of the synaptic response averaged 100.3% ± 6.3% and 98.8% ± 7.6% of their control amplitudes prior to the tetanus, respectively (Fig. 3e). In addition, the effect of this peptide seems to be specific, since it has been shown that although intradendritic injections of the PKA inhibitory peptide PKI5–24 (see Fig. 3d) blocked the dopamine-mediated potentiations (24), they did not block induction of the activity-dependent enhancements [in a preliminary series, n = 5, PKI5–24 successfully prevented dopamine-evoked potentiations but was unable to block induction of activity-dependent potentiations (S. Kumar and D. S. Faber, personal communication)].

To further investigate the regulatory role of CaM-KII, we next determined whether intradendritic injections of a constitutively active form of CaM-KII could potentiate synaptic...
responses, as occurs in region CA1 of hippocampus (10). Indeed, CaM-KII injection resulted in slow increases in both components of the synaptic response to approximately 150% of control (Fig. 4a–c). On the other hand, injecting a heat-inactivated form of this kinase did not increase the synaptic responses. M-cell antidromic spike did not change significantly in both experimental conditions (Fig. 4c). These enhancements did not saturate or plateau, which prevented us from examining whether the CaM-KII-induced poten- tiations occluded those evoked by tetanic stimulation.

Immunohistochemistry using the G-301 antibody, which ex- hibits relative selectivity for the α-subunit of rat brain CaM-KII (Fig. 5a–c), demonstrated that CaM-KII was present in the M-cells, particularly in their lateral dendrites (Fig. 5c), where these afferent synapses are localized. Immunoblot analysis of goldfish brain homogenate with G-301 revealed the presence of multiple immunoreactive bands of a size similar to the rat brain α-subunit (Fig. 5d). Studies using a second antibody, RU-16, directed against the C terminus of the β-subunit of rat CaM-KII, showed comparable results (data not shown).

![Fig. 4](image_url)

**Fig. 4.** Intradendritic injections of a constitutively active form of α-CaM-KII enhanced both components of the synaptic response. (a) Time course of two experiments in which recordings were obtained with electrodes containing either the constitutively active or a heat-inactivated form of α-CaM-KII. (b) Superimposed average traces (>20) obtained at different intervals after penetrating the dendrite with an electrode containing α-CaM-KII. Typically, leakage of α-CaM-KII alone caused continuously increasing potentiations. (c) Bar plots represent the normalized amplitudes of the synaptic components and antidromic (AD) spike measured at the end of the recording sessions in experiments with α-CaM-KII (30–80 min; electrical and chemical PSPs averaged 152.1% ± 6.5% and 158.3% ± 16.7% of their respective control amplitudes, respectively, n = 7) and heat-inactivated α-CaM-KII (40–80 min; corresponding synaptic responses averaged 98.3% ± 6.6% and 96% ± 7.4%, n = 7). M-cell antidromic spike height did not change significantly in either experimental series.

![Fig. 5](image_url)

**Fig. 5.** CaM-KII immunoreactivity is present in goldfish M-cells. (a–c) Immunohistochemical evidence for the presence of CaM-KII in the M-cell. Confocal (pseudocolor; white corresponds to maximum bright- ness) images obtained with the antibody G-301 (1:1000; Texas red). (a) Soma. (Bar: 50 μm.) (b) Lateral dendrite. (Bar: 30 μm.) (c) Higher magnification view of another stained lateral dendrite. (Bar: 15 μm.) Note the punctate staining surrounding the M-cell soma and lateral den- drite, most likely corresponding to the precortical localization of this enzyme. (d) Immunoblot using G-301 (1:200 dilution) and the following: lane a, 2 μg of rat cerebral cortex homogenate; lane b, 2.5 ng of CaM-KII purified from rat forebrain (11); lanes c and d, 30 μg and 90 μg, respectively, of goldfish brain homogenate. Molecular weight markers (× 103) are shown on the left. (e) Schematic representation of the proposed potentiating pathway. KII, CaM-KII; R, receptor.
Our data show that a postsynaptic elevation of Ca\(^{2+}\) concentration is an essential step for the induction of activity-dependent potentiation of the eighth nerve mixed synaptic responses. Although there could be other sources, this increase in Ca\(^{2+}\) is likely to be synthetically mediated, is probably localized to the postsynaptic densities (possibly forming a NMDA receptor microdomain), and leads to the activation of CaM-KII, which seems to be essential for the induction process (Fig. 5e). Because the changes are relatively rapid, and potentially transient, CaM-KII is also likely to be involved in at least the early phase of the expression of the potentiations. Likely targets of CaM-KII (Fig. 5e) are non-NMDA glutamate receptors (25, 26) and gap junction proteins (27). Although the mechanism of action of CaM-KII on gap junction channels is presently unknown, it is likely to involve changes in single channel conductance and/or open probability, as is the case for other kinases (28).

Multifunctional CaM-KII has a well established role in modulating chemical synaptic plasticity (16–18). The present results show that its modulatory role is not restricted to a specific modality of synaptic transmission, and they suggest a primary role for CaM-KII in shaping and regulating interneuronal communication in general. In the case of the M-cell system, the increased synaptic gain of these eighth nerve synapses will sensitize a vital escape response, lowering its threshold to auditory stimuli.

Finally, in contrast to the generally accepted tenet, elevations of intracellular Ca\(^{2+}\) concentration can lead to an increase in gap-junctional conductance. This effect is synthetically mediated and asymmetric—i.e., restricted to only one side of the gap junction plaque. This finding suggests that the hemichannels in the membrane of the postsynaptic M-cell are likely to be different in connexin composition from those in the presynaptic side (heterologous gap junctions; refs. 29 and 30) or that there may be different regulatory mechanisms in operation on the two sides of the connection, namely the M-cell dendrite and the afferent terminals. From the functional point of view, this asymmetric regulatory phenomenon implies that each individual neuron may be capable of exerting autonomous control of the degree of intercellular communication with its neighbors. This strategy could be essential for the rapid and tight regulatory control of these gap junctions in the M-cell system.

The gap junction regulatory mechanism described here may constitute a widespread property of electrical synapses that can apply to these junctions in other tissues. As an example, a synthetically mediated unilateral control mechanism may be relevant during neural development, where both gap-junctional coupling and activity-dependent synaptic plasticity are believed to play essential roles in establishing connections and creating functional compartments (31, 32).

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