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Protein kinase A-independent modulation of ion channels in the brain by cyclic AMP

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ABSTRACT **Ion channels underlying the electrical activity of neurons can be regulated by neurotransmitters via two basic mechanisms: ligand binding and covalent modification. Whereas neurotransmitters often act by binding directly to ion channels, the intracellular messenger cyclic AMP is thought usually to act indirectly, by activating protein kinase A, which in turn can phosphorylate channel proteins. Here we show that cyclic AMP, and transmitters acting via cyclic AMP, can act in a protein kinase A-independent manner in the brain. In hippocampal pyramidal cells, cyclic AMP and norepinephrine were found to cause a depolarization by enhancing the hyperpolarization-activated mixed cation current, I_Q (also called I_h). This effect persisted even after protein kinase A activity was blocked, thus strongly suggesting a kinase-independent action of cyclic AMP. The modulation of this current by ascending monoaminergic fibers from the brainstem is likely to be a widespread mechanism, participating in the state control of the brain during arousal and attention.**

Neurotransmitters can regulate neuronal excitability by activating second-messenger pathways leading to covalent modifications of ion-channel proteins. In particular, a number of transmitters exert their effects by activating cyclic AMP (cAMP) production. cAMP activates protein kinase A (PKA), which in turn can phosphorylate ion channels and other target proteins (1–3). In neurons, this enzyme has so far been regarded as the main intracellular receptor for cAMP (1). However, in a few sensory (4) and muscle (5–7) cells, cAMP has also been found to have direct, PKA-independent effects on some ion channels.

In CA1 hippocampal neurons, cAMP and the monoamine transmitters activating cAMP production—norepinephrine, serotonin, histamine, and dopamine—are all known to increase excitability by suppressing the Ca^{2+}-activated K^+ current, I_{AHP} (8–16), which is a current underlying the slow afterhyperpolarization (sAHP) and spike frequency adaptation (17). The suppression of I_{AHP} has previously been shown to be mediated by PKA for each of these monoamine transmitters (10, 16).

We now address the question whether cAMP and the transmitters acting via cAMP can also exert kinase-independent effects in these neurons. A possible candidate for such an unconventional cAMP effect is the depolarization of the membrane potential that accompanies the suppression of I_{AHP} in response to cAMP and monoamine transmitters (8, 11, 12). We found that the depolarizing action of cAMP and β-adrenergic agonists was due to an enhancement of the cation current I_Q (also called I_h; refs. 18 and 19) and that this effect persisted even when the activation of PKA, and the associated suppression of I_{AHP}, was blocked. Thus, it seems that cAMP can enhance the excitability of these neurons by modulating ion channels in a PKA-independent manner.

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MATERIALS AND METHODS

Transverse hippocampal slices (400 μm thick) were prepared from young Wistar rats (16–28 days old) that were decapitated under halothane anaesthesia. During recording, the slices were superfused with extracellular medium (125 mM NaCl/25 mM $NaHCO_3$/1.25 mM KCl/1.25 mM KH_2PO_4/2.0 mM $CaCl_2$/1.5 mM $MgCl_2$/16 mM glucose, saturated with 95% O_2/5% CO_2) at 23–26°C. Bicuculline (10 μM) was routinely added to the medium to suppress spontaneous inhibitory synaptic currents. In voltage clamp experiments, tetrodotoxin (0.5 μM) was added to the bath to block Na^+ channels, and in experiments designed for recording I_{AHP}, tetraethylammonium (TEA, 5 mM) was also added. Whole-cell GΩ-seal recordings were obtained "blindly" from CA1 pyramidal cells, using an Axopatch 1D amplifier (2-kHz low-pass filter) for both current-clamp and voltage-clamp recordings. The patch pipettes were filled with a solution containing 140 mM potassium gluconate, 10 mM Hepes, 2 mM ATP, 3 mM $MgCl_2$, and 0.4 mM GTP (pipette resistance, 5–8 MΩ). In some experiments designed to measure Ca^{2+}-independent processes, 5 mM EGTA and 3 mM $CaCl_2$ were added to the internal solution. To elicit I_{AHP}, a depolarizing step (100 ms) of sufficient amplitude to elicit a robust (unclamped) Ca^{2+} action current was applied once every 30 s (10). Series resistance compensation was not used. The access resistance (range, 10–30 MΩ) showed only minimal variations during each recording. All membrane potential values have been nearly fully corrected (by 10 mV) for the liquid junction potential measured between the extracellular and the intracellular media (+ 11 mV). Substances were generally applied by adding them to the superfusing medium. Stock solutions of norepinephrine or isoproterenol were kept frozen to avoid oxidation. The PKA peptide inhibitor (PKI) was included in the intracellular medium and allowed to diffuse into the cell for at least 20 min before testing with isoproterenol. When PKI was used, the isoproterenol application was often prolonged to ensure that the lack of effect on I_{AHP} was not due to insufficient time for diffusion.

All drugs were obtained from Sigma, except tetrodotoxin and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), which were from Research Biochemicals (Natick, MA), and the PKI peptide (Walsh peptide; sequence, TTYADFIASGRT-GRRNAIHD-amide), which was synthesized in Paul Greengard's laboratory at Rockefeller University.

RESULTS AND DISCUSSION

Whole-cell recordings were obtained from CA1 pyramidal cells (n = 80) in rat hippocampal slices. Norepinephrine and

Abbreviations: PKA, protein kinase A; PKI, PKA inhibitor; sAHP, slow afterhyperpolarization; 4-AP, 4-aminopyridine; 8CPT-cAMP, 8-(4-chlorophenyl)thioadenosine 3′,5′-(cyclic)-monophosphate; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; TEA, tetraethylammonium.

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β-adrenergic receptor agonists had two effects in hippocampal neurons (8): (*i*) depolarization of the membrane (Fig. 1*a1*), corresponding to an inward current in voltage clamp (Fig. 1*e*) and (*ii*) inhibition of the sAHP (Fig. 1*a2*) and the underlying current, I_{AHP} (Fig. 1*c2*). These effects are clearly mediated by β-adrenergic receptors (8), as they were resistant to α-adrenergic receptor antagonists (Fig. 1*a*), mimicked by the β-adrenergic agonist isoproterenol (n = 6; Fig. 1 *b* and *c*), and blocked by β-adrenergic receptor antagonists (timolol, n = 5; propranolol, n = 3; data not shown).

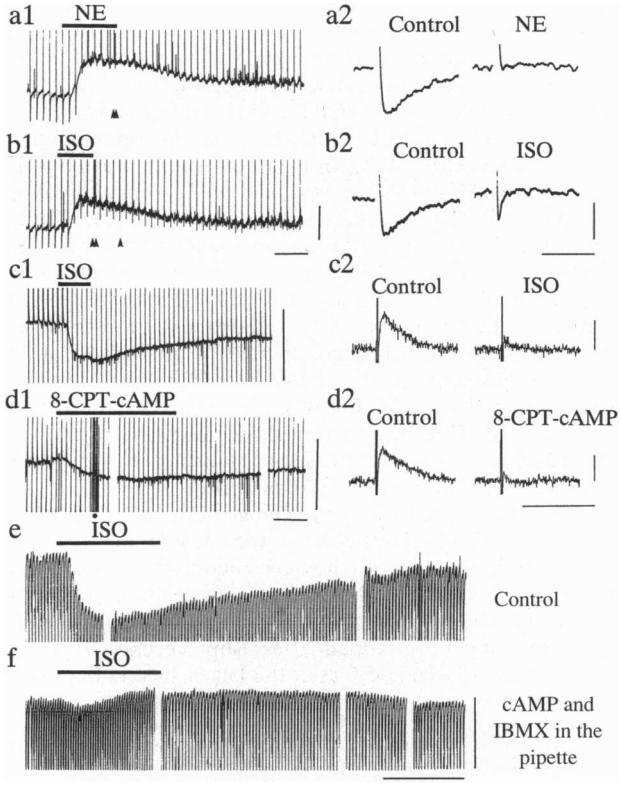

FIG. 1. Norepinephrine depolarizes hippocampal neurons by eliciting an inward membrane current via β-adrenergic receptors and cAMP. Whole-cell recordings were made from CA1 hippocampal pyramidal cells in brain slices from rats: membrane potential recordings (current clamp) (*a* and *b*) and membrane current recordings in voltage clamp (*c–f*). Holding potential (V_h), −70 mV. (*a*) Norepinephrine (NE, 2.5 μM) caused a depolarization (*a1*) and inhibition of the sAHP following a spike burst, in the presence of the α-adrenergic receptor blocker phentolamine (20 μM). The vertical lines in *a1* and *b1* represent bursts and AHPs elicited by injecting a 100-ms depolarizing current pulse once every 30 s (samples in *a2* and *b2*). Arrowheads mark spontaneous action potentials. (*b*) The selective β-adrenergic receptor agonist isoproterenol (ISO, 5 μM) caused a similar depolarization (*b1*) and inhibition of the sAHP (*b2*). (*c*) Voltage-clamp recordings of the inward current underlying the depolarizing response to isoproterenol (ISO, 5 μM) (*c1*) and inhibition of the AHP current (I_{AHP}) (*c2*). V_h, −70 mV. (*d*) Similar effects of bath application of the membrane-permeant cAMP analogue 8-(4-chlorophenyl)thioadenosine 3′,5′-(cyclic)-monophosphate (8CPT-cAMP; 500 μM) in the presence of the adenosine receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 20 μM) to prevent side effects of 8CPT-cAMP. The effect of 8CPT-cAMP was slower than that of isoproterenol, presumably due to slow diffusion, but amplitude was similar for the two drugs. (Note the different scale bars in *c1* and *d1*: 20 pA and 40 pA, respectively.) A series of hyper- and depolarizing pulses (for *I–V* plot) were given during the responses (black dot in *d1*). Subsequent *I–V* series were deleted for clarity (gaps in records *d–f*; similar in Figs. 2–4). (*e* and *f*) Intracellular cAMP occluded the depolarizing effect of isoproterenol. In a control cell isoproterenol (10 μM) had full effect (*e*), whereas the same dose caused almost no inward current in a cell recorded with 5 mM cAMP (5 mM) and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 8 μM) in the pipette for 15 min (*f*). In *e* and *f*, 20-mV hyperpolarizing pulses (1 s) were given once every 3–4 s to monitor changes in membrane conductance and voltage-gated currents (see Fig. 4). Scale bars: 4 mV, 100 s (*a1* and *b1*); 4 mV, 2 s (*a2* and *b2*); 20 pA (*c1*); 40 pA (*d1*), 200 s (*c1* and *d1*); 20 pA, 5 s (*c2* and *d2*); 20 pA, 100 s (*e* and *f*).

The inward current (n = 6) and the inhibition of I_{AHP} (n = 12) were mimicked by the membrane-permeant cAMP analogue 8CPT-cAMP (Fig. 1 *d1* and *d2*) (9) and were occluded by intracellular application of cAMP and a phosphodiesterase inhibitor (n = 3; Fig. 1*f*) or by prior bath application of 8CPT-cAMP (n = 3; data not shown). These results indicate that cAMP mediates both effects (9), as expected, since β-adrenergic receptors stimulate cAMP production in the brain (20).

How does cAMP produce these effects? The effects of cAMP are generally mediated by PKA (21). However, cAMP has also been found to have direct, PKA-independent effects in sensory (4) and muscle (5–7) cells, but not in central nervous system neurons. To test whether the β-adrenergic receptor- and cAMP-induced depolarization was also PKA-dependent, we used intracellular application of a highly selective and potent inhibitor of PKA: the pseudosubstrate PKI (Walsh peptide), which binds to the catalytic site of PKA (22). In the five cells tested, the PKI peptide failed to reduce the depolarizing current induced by isoproterenol (Fig. 2 *b* and *c*). In contrast, the PKA-dependent modulation of I_{AHP} (10) by isoproterenol was suppressed in the same cells (Fig. 2 *b* and *d*),

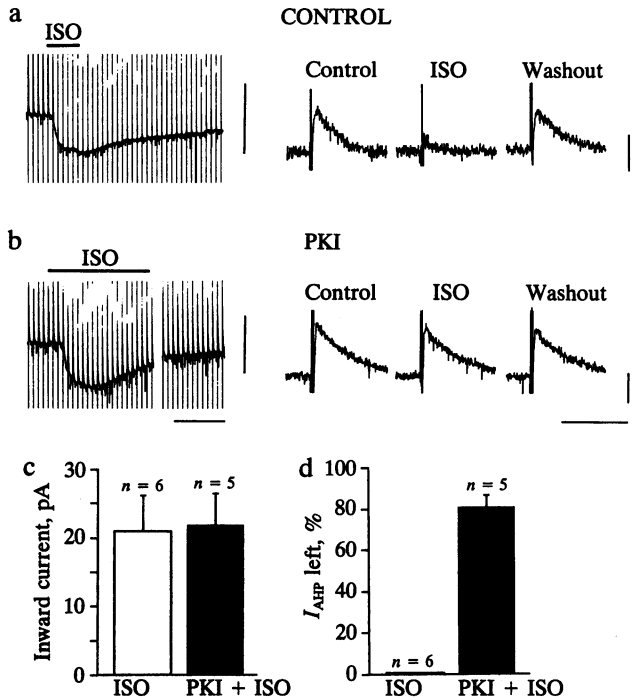

FIG. 2. Inhibition of PKA does not reduce the inward current elicited by β-adrenergic receptor activation in CA1 cells. (*a*) Under normal conditions, isoproterenol (ISO, 5 μM) elicited an inward current (*Left*) and suppressed I_{AHP} in the same cell (*Right*). (*b*) In a cell dialyzed with PKI (1 mM), a potent and specific inhibitor of PKA, isoproterenol still elicited an inward current but had essentially no effect on I_{AHP} in the same cell, indicating that PKA was blocked. Scale bars: 20 pA, 5 min (*Left*); 20 pA, 5 s (*Right*). The vertical lines in records *a* and *b* (*Left*) represent depolarizing pulses used to elicit I_{AHP} once every 30 s. (*c*) Summary of the results from all cells tested with isoproterenol (ISO, 3–5 μM) with (n = 5) or without (n = 6) PKI (1 mM) in the pipette. The peak amplitude of the inward current elicited by isoproterenol was similar with and without PKI. (*d*) In contrast, the suppression of I_{AHP} (given as the minimal remaining I_{AHP} amplitude in percent of the amplitude just before isoproterenol was applied) was largely prevented by PKI. V_h, −70 mV in *a–d*.

indicating an efficient blockade of PKA (10). This observation strongly suggests that the inward current is independent of PKA, whereas it is clearly mediated by cAMP (Fig. 1) (9). This is, to our knowledge, the first indication that cAMP has a direct, PKA-independent effect in the brain, and also the first example of a bifurcating cAMP signal pathway, with both PKA-dependent (AHP modulation) and PKA-independent (depolarization) mechanisms operating in the same neuron. This conclusion is further supported by the observation that the inward current evoked by another cAMP analogue (9, 23, 24) was not blocked by the nonselective kinase inhibitor staurosporine (23, 24).

We next asked which ionic mechanism underlies the inward current induced by isoproterenol or 8CPT-cAMP. In all cells tested, this current was blocked by bath application of 1–4 mM Cs^+ ($n = 6$; Fig. 3*e*), which itself caused an outward shift in the holding current (Fig. 3*e*) (18, 19). In contrast, other ion channel blockers failed to prevent the inward current: TEA (5–10 mM; $n = 14$; Fig. 3*b*), which blocks the delayed rectifier K^+ current, the M current (I_M), and the C current; 4-AP (1–2 mM; $n = 4$; Fig. 3*c*), which blocks the A and D currents; or Ba^{2+} (1 mM; $n = 4$; Fig. 3*d*), which blocks I_M and the fast, K^+-selective inward rectifier conductance and a resting K^+ conductance (25, 26). The inward current induced by isoproterenol was also resistant to tetrodotoxin (0.2–1 μM; $n = 30$; Figs. 2–4) and Ca^{2+}-free medium, which eliminates Ca^{2+}-dependent currents ($n = 10$; Fig. 3 *a–f*).

This pharmacological profile points to the Q current (I_Q, also called I_h) (18, 19), which is the only known current in hippocampal neurons that is sensitive to Cs^+ but not to Ba^{2+}, TEA, or 4-AP (18, 19, 25, 26). I_Q is due to voltage-gated cation channels that are opened slowly by hyperpolarization and are permeable to Na^+ and K^+ ions, producing an inward (depolarizing) current at the resting potential (18, 19). Similar currents in other cells are called I_h or I_{AR} (27–29) or I_f (30–32).

If I_Q underlies the β-adrenergic receptor- and cAMP-induced inward current, one would expect that isoproterenol or 8CPT-cAMP should enhance I_Q. In particular, there should be an increase in the activation of I_Q during a step from −50 mV, where I_Q is not (or only minimally) activated (18, 19), to the resting potential, where the depolarizing current is normally observed. As expected, when stepping from −50 mV to −70 mV, isoproterenol or 8CPT-cAMP caused an increase in the slow inward current, which reflects the opening of *Q* channels, in all cells tested (for isoproterenol, $n = 10$, Fig. 4*c1* and *c3*; for 8CPT-cAMP, $n = 4$, Fig. 4*c2*). Current–voltage (*I–V*) plots also showed that isoproterenol induced the expected increase in the instantaneous conductance when the cell was held at a potential where I_Q is active (−70 mV in Fig. 4*g*), presumably reflecting an increased number of open Q channels at this potential. Fig. 3*f* also illustrates that the depolarizing current was accompanied by an increase in the membrane conductance at −70 mV, where the Q channels are open, but not at −50 mV (Fig. 4*a*), where most Q channels are closed ($n = 6$). Thus, the current deflections due to ±10 mV voltage clamp steps were increased by isoproterenol, indicating a conductance increase at −70 mV (Fig. 3*f*). In contrast, isoproterenol caused little or no change in the holding current (Fig. 4*a*) or instantaneous conductance at −50 mV (Fig. 4*c*), whereas the I_Q relaxations (the dark band marked with brackets in Fig. 4*a*) increased with a time course similar to that of the inward current at −70 mV (Fig. 4*b*). The I_Q hypothesis also implies that if Cs^+ is added after isoproterenol, it should reverse the depolarizing effect of the latter, by eliminating I_Q.

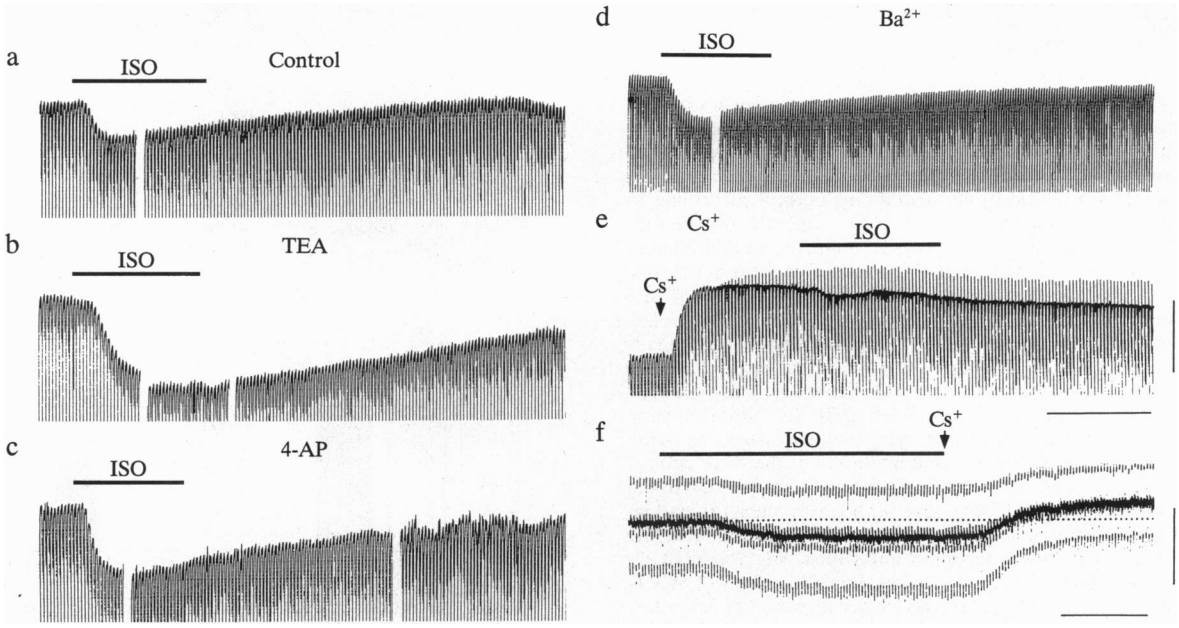

FIG. 3. The inward current evoked by β-adrenergic receptor activation is blocked by Cs^+ and is associated with an increase in membrane conductance. (*a*) Response to isoproterenol (ISO, 10 μM) under normal conditions. The downward deflections in *a–e* are responses to hyperpolarizing steps given once every 5 s to monitor voltage-gated currents (see Fig. 4). (*b–d*) Responses to isoproterenol (ISO, 5–10 μM) in the presence of 10 mM TEA (*b*), 1 mM 4-aminopyridine (4-AP) (*c*), and 1 mM $BaCl_2$ (*d*). None of these K^+ channel blockers prevented the inward current evoked by isoproterenol. Each test was performed in a different cell, to avoid β-adrenergic receptor desensitization. The variability between cells (*a–d*) was about the same in TEA, 4-AP, Ba^{2+}, and under control conditions. (*e*) CsCl (1 mM, arrow) caused an outward shift in the membrane current, and prevented the effect of a subsequent application of isoproterenol (ISO, 10 μM). The Cs^+ application started at the arrow and continued for the length of the recording. The slow inward drift in the holding current may reflect intracellular Cs^+ accumulation. The overshooting peaks that appear in Cs^+ are due to outward transients uncovered by block of the I_Q relaxations. (*f*) Isoproterenol causes an inward current and conductance increase, which are reversed by Cs^+. To monitor the input conductance, depolarizing and hyperpolarizing steps (±10 mV × 100 ms) were given once every 1 s. The amplitudes of the current responses are seen as bands above and below the holding-current trace; the separation between these bands reflects the conductance. Application of 1 mM Cs^+ (arrow) rapidly reversed the effects of isoproterenol (ISO, 5 μM) and caused an additional outward shift and conductance decrease. The amplification in *f* is one-fifth of that in *a–e*, and the time scale is 4 times faster. Scale bars: 20 pA, 2 min (*a–e*); 100 pA, 30 s (*f*). In *a–f*, $V_h = -70$ mV; with 0 mM Ca^{2+}, 2.5 mM $MnCl_2$, and 0.5 μM tetrodotoxin in the bath.

This prediction was also confirmed (Fig. 3*f*, arrow). Cs^+ also caused a net outward shift in the holding current beyond the control level prior to isoproterenol (dotted line in Fig. 3*f*), as expected, since I_Q is partly activated at −70 mV (19).

A plausible mechanism for the I_Q-dependent depolarization is a shift to less negative potentials in the voltage dependence of I_Q (5, 6, 29, 32), causing it to be more strongly activated near the resting potential. Such a positive shift in the activation curve would be expected to be accompanied with a change in the activation kinetics (29, 32). In accordance with this hypothesis, the time constant of activation was shortened by isoproterenol (n = 3; Fig. 4*d*): in each of the three cells tested, the time constant was reduced at all voltages tested (altogether in 13 of 13 tests). The average reduction was 9.8 ± 1.3% (range, 5.9–13.6% for the 3 cells). Finally, currents evoked by ramp commands from +10 mV to −130 mV (Fig. 4*e*), as well as I–V plots of the I_Q relaxation (Fig. 4*f*), showed the expected increase in inward current at potentials negative to −60 mV, corresponding to the activation range of I_Q. The I_Q relaxations elicited by voltage steps from −50 to −70 mV in the presence of 1 mM Ba^{2+} showed an increase of 23 ± 10.6 pA after application of isoproterenol (n = 3). Under the same conditions, isoproterenol elicited a depolarizing inward current of 27.3 ± 6.9 pA in cells voltage-clamped at −70 mV (n = 3). The changes in I_Q therefore seem sufficiently large to account for the depolarization and inward current at −70 mV in response to β-adrenergic receptor agonists.

In summary, the data presented here indicate that norepinephrine, acting via β-adrenergic receptors and cAMP, produces a direct, PKA-independent modulation of the cation current I_Q (I_h), causing a stronger activation close to the resting potential. The resulting depolarization increases the excitability of the target cell and can trigger action potentials (Fig. 1 *a1* and *b1*; arrowheads). This mechanism adds to the excitability increase caused by inhibition of I_{AHP}, and both actions are likely to contribute to the noradrenergic modulation of hippocampal activity.

Other monoamine transmitters also stimulate cAMP production and cause depolarization and inhibition of the sAHP in hippocampal neurons (3), including serotonin (11, 12),

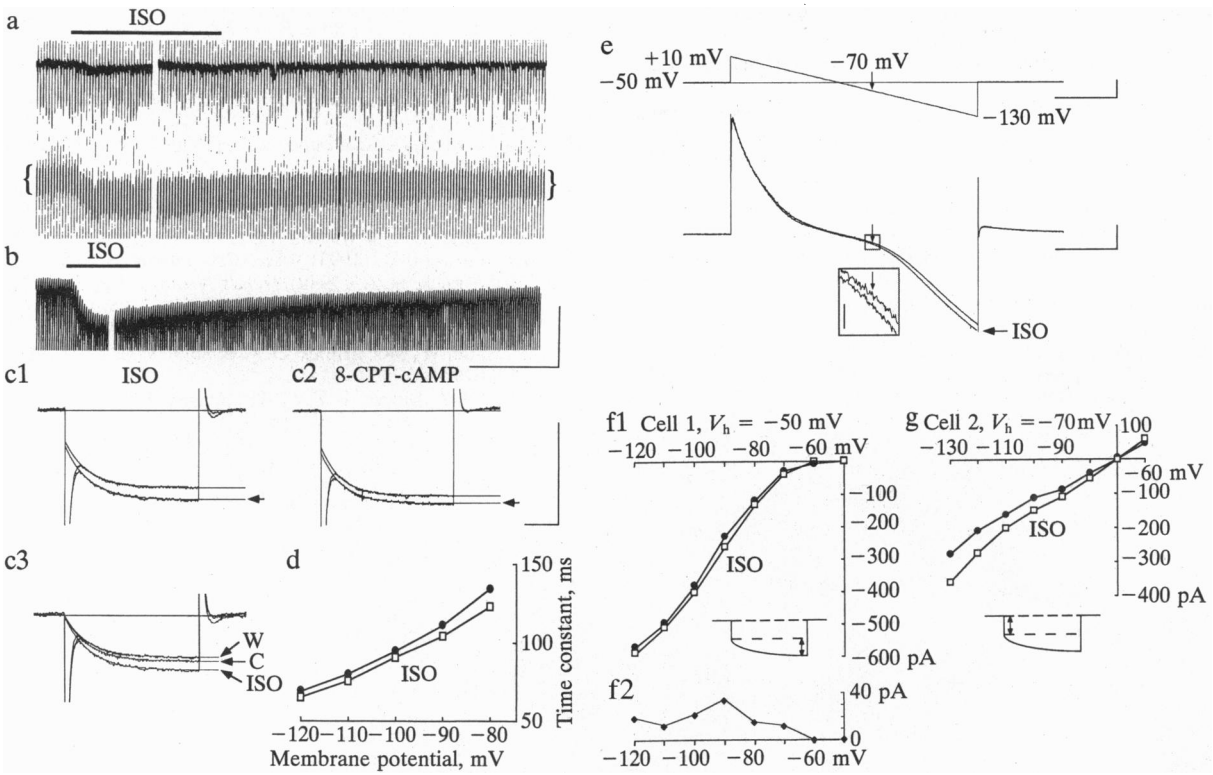

FIG. 4. β-Adrenergic receptor activation and a cAMP analogue enhance the *Q* current (I_Q), thus causing the depolarizing current near the resting potential. (*a* and *b*) Comparison between the effects of isoproterenol (ISO, 5–10 μM) at two different V_h, −50 mV and −70 mV. (*a*) At −50 mV, where I_Q is not activated, isoproterenol caused virtually no change in the holding current but increased the inward current evoked by hyperpolarizing steps to −70 mV (duration, 1 s). The I_Q relaxations during these steps (samples shown in *c1–c3*) form a dark band, marked with brackets, which was widened by isoproterenol, reflecting an increase in I_Q. (The band does not show the full increase in I_Q, because the capacitative transients mask more of the relaxation when its rate of activation is increased; see *c* and *d*.) (*b*) At −70 mV, the time course of the isoproterenol-induced inward current resembles that of the increase in I_Q relaxations (marked with brackets in *a*), as expected if I_Q underlies the inward current at −70 mV. (*c*) Isoproterenol (5 μM) (*c1*) or 8CPT-cAMP (500 μM) (*c2*) enhanced I_Q (arrows) elicited by a step from −50 mV to −70 mV. In both cases, there was an increase in the slow inward current reflecting the opening of Q channels. (*c3*) Records before (control, C) and during application of isoproterenol (ISO, 5 μM), and after washout (W); here the ohmic component of the leak (but not the capacitive transients) has been subtracted. Each current trace in *c1–c3* has been fitted with a single exponential [equation: $y(t) = a \cdot \exp(-t/\tau) + b$]. (*d*) Isoproterenol (5 μM) reduced the time constant of I_Q activation. (*e*) Current evoked by a voltage ramp command from +10 to −130 mV, before and during application of isoproterenol (ISO, 10 μM), which caused an increase in inward current negative to −60 mV, corresponding to the activation range of I_Q. The change in current at −70 mV was 12 pA in this case (*Inset*)—i.e., similar to the isoproterenol-induced current increase during steps to −70 mV, in the same cell (*a*). (*f* and *g*): Current–voltage (*I–V*) plots from a series of 1-s-long voltage-clamp steps from two different V_h, −50 mV (*f*) and −70 mV (*g*), in two different cells. (*f1*) *I–V* plot of the Q current relaxation (*Inset*), showing an increase by isoproterenol negative to −50 m. (*f2*) Plot of the net increase in the I_Q relaxation after isoproterenol (i.e., the difference between the plots in *f1*). (*g*) *I–V* plot (from another cell; V_h, −70 mV) of the instantaneous current before and after isoproterenol (10 μM), showing an increase in the instantaneous conductance at −70 mV. The data in *a*, *c1–c3*, *e*, and *f* are from the same cell; *b*, *d*, and *g* are from other cells, all recorded in Ca^{2+}-free medium with 2.5 mM Mn^{2+} and 0.5 μM tetrodotoxin. Plots in *d*, *f*, and *g*: control, ●; isoproterenol, □. Scale bars: 20 pA, 2 min (*a* and *b*); 100 pA, 200 ms (*c1–c3*); 40 mV, 5 s (*e Upper*); 200 pA, 5 s (*e Lower*); 25 pA (*e*, *Inset*).

histamine (13), and dopamine (14, 15). Hence, it seems likely that these transmitters also induce modulation of I_h by cAMP. The same may be true in the thalamus (29) and other regions where modulation of I_h is thought to be of importance for the state control of the brain (33–35). Thus, the mechanism described here may participate in the activation of the forebrain during arousal and attention (8, 33–36).

A similar mechanism has previously been reported to underlie the acceleration of the heartbeat by adrenaline, which also is mediated by a direct cAMP effect on the same kind of cation current (5, 6, 32). Thus, there seems to be a striking parallel between the molecular mechanisms operating in the brain and in the heart during arousal.

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