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Long-term regulation of synaptic acetylcholine release and nicotinic transmission: the role of cyclic AMP

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1 Using the rat superior cervical ganglion *in vitro*, the relative efficacy of nicotinic synaptic transmission was estimated by recording the postganglionic compound action potential and the amount of endogenous acetylcholine (ACh) released. These two parameters were correlated in individual ganglia by sampling the bathing medium for the assay of ACh while simultaneously recording the postganglionic response.

2 The β-adrenoceptor agonist isoprenaline potentiated both the evoked release of ACh and the postganglionic response by about 20% during preganglionic stimulation at 0.2 Hz.

3 The adenosine receptor agonist 2-chloroadenosine inhibited ACh release and the postganglionic response by about 35%.

4 Tetanic preganglionic stimulation for a few seconds induced a long-term potentiation of nicotinic responses and of ACh release. Both of these potentiations were dependent upon extracellular Ca^{2+} during the tetani.

5 Forskolin and analogues of cyclic AMP also caused a long-lasting potentiation of both the evoked release of ACh and the postganglionic response, indicating that cyclic AMP may regulate transmission by a presynaptic mechanism. The specificity of the cyclic AMP analogues was tested using various butyryl- and bromo-purine nucleotides.

6 The effects of forskolin and 8-bromo-cyclic AMP did not appear to be dependent upon extracellular Ca^{2+}.

7 The potentiation caused by forskolin was consistently augmented by three phosphodiesterase inhibitors – AH 21-132, papaverine and SQ 20-006. However, the effect of forskolin was not consistently enhanced by theophylline, nor was it reduced by the adenylate cyclase inhibitor SQ 22-536.

8 The neurogenic long-term potentiation was augmented by two of the phosphodiesterase inhibitors that also augmented the forskolin-induced potentiation – papaverine and SQ 20-006.

9 It was concluded that cyclic AMP can enhance nicotinic transmission, and can do so by increasing the evoked release of ACh. However, it was not possible to prove that cyclic AMP mediates the long-term potentiation induced by tetanic preganglionic stimulation.

Introduction

Nicotinic synaptic transmission in autonomic ganglia is subject to a variety of regulatory processes. Some of these processes can be affected by extracellular substances. For example, β-adrenoceptor agonists potentiate and adenosine receptor agonists inhibit nicotinic transmission in the sympathetic ganglion (Brown & Dunn, 1983a,b; Henon & McAfee, 1983a). Here we demonstrate that these substances likewise alter the evoked release of endogenous acetylcholine (ACh) from the ganglion.

In other instances, synaptic transmission can be regulated by use-dependent processes within the ganglion. One remarkable example is long-term potentiation (l.t.p.), in which a burst of preganglionic activity lasting a few seconds induces an hours-long potentiation of transmission (Briggs *et al.*, 1985a). This too was found to be accompanied by a long-term potentiation of the evoked release of acetylcholine (Briggs *et al.*, 1985b).

The persistence of l.t.p. and the brevity of its

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induction by tetanic stimulation suggests that the formation of this potentiation is mediated by a metabolic process. The nature of that process is unclear. In the hippocampus, it has been proposed that l.t.p. is mediated by adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Stanton & Sarvey, 1985a,b), or by Ca^{2+} phospholipid-dependent protein kinase C (Akers *et al.*, 1986; Malenka *et al.*, 1986), or by a Ca^{2+}-dependent protease (Lynch & Baudry, 1984). Studies in invertebrates clearly demonstrate neuronal modulation by cyclic AMP-dependent protein kinase, by Ca^{2+}/calmodulin-dependent protein kinase, and by protein kinase C (see Kandel & Schwartz, 1982; Abrams *et al.*, 1984; Levitan, 1985; Byrne, 1987).

We undertook the present study to determine if nicotinic synaptic transmission in the sympathetic ganglion is regulated by a cyclic AMP-dependent process, and if nicotinic l.t.p. is a consequence of this process.

Methods

Superior cervical sympathetic ganglia were removed from Sprague-Dawley rats (170–220 g, of either sex) killed by decapitation. The ganglia were desheathed and maintained at 22–25°C in a medium similar to Locke solution. The medium was continuously gassed with 95% O_2, 5% CO_2 and contained the following (in mM): NaCl 136, KCl 5.6, $CaCl_2$ 2.2, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 20 and dextrose 8 (McAfee, 1982). In some experiments, a low-Ca^{2+} solution was made by omitting the $CaCl_2$ and increasing the $MgCl_2$ to 8.0 mM. These bathing media were continuously superfused (1 ml min^{-1}), except during the collection of samples for acetylcholine and choline measurement.

Measurement of the postganglionic response

In the experiments where only the postganglionic compound action potential was measured, the techniques and protocols were similar to those described previously (Briggs *et al.*, 1985a). The ganglion was placed in a 1 ml chamber fitted with suction electrodes and continuously superfused with oxygenated Locke solution containing atropine (2 μM) to block muscarinic transmission, and (+)-tubocurarine (50–100 μM) or hexamethonium (50–200 μM) to inhibit partially nicotinic transmission so that the postganglionic response was submaximal. In some experiments, exogenous choline (10 μM) was added as a substrate for acetylcholine (ACh) synthesis. The preganglionic cervical sympathetic nerve was stimulated with isolated monophasic current pulses (500 μs duration) at an intensity 1.5 fold greater than that required to elicit a maximal postganglionic response. The evoked postganglionic compound action potential was recorded from the internal carotid nerve and measured by a computerized data acquisition system.

Stimulus and testing conditions

The ganglion was stimulated continuously with one preganglionic shock per minute (0.017 Hz) when only the postganglionic response was measured, or with one shock per 5 s (0.2 Hz) when the release of ACh was also measured. This constant test stimulation was interrupted only by the brief preganglionic tetani (5–20 Hz for 5–20 s) used to induce nicotinic long-term potentiation (l.t.p.). In some experiments, brief exposure to a drug was used in place of tetanic stimulation to induce a long-lasting potentiation.

Measurement of acetylcholine and choline release

The release of ACh or choline was measured simultaneously with the postganglionic response, using procedures similar to those described previously (Briggs *et al.*, 1985b). The ganglion was placed in a 65 μl chamber fitted with suction electrodes and superfused with Locke solution containing atropine (2 μM), (+)-tubocurarine (50–100 μM) or hexamethonium (100–200 μM), and, unless otherwise indicated, physostigmine (20 μM) to inhibit cholinesterase.

After 2 h, samples of the bathing medium were collected for assay of ACh and choline in the absence of any stimulation (spontaneous release), and during continuous supramaximal stimulation of the preganglionic nerve (0.2 Hz). During the sample collection, superfusion was stopped in order to maximize the recovery of the released ACh. The bath was gently stirred and oxygenated by bubbling 95% O_2, 5% CO_2. Samples were obtained at 5 min intervals by withdrawing 50 μl of the bath and replacing it with fresh oxygenated medium, effectively exchanging the bath. The samples were frozen immediately on dry ice and then stored overnight at −20°C for subsequent assay, except the samples collected during the first 25 min of stimulation at 0.2 Hz which were discarded.

Assay of acetylcholine and choline

The radioenzymatic procedure originally described by Goldberg & McCaman (1974) and subsequently modified by McCaman & Stetzler (1977) was used. Each 50 μl sample of the bathing medium was first extracted with chloroform to remove the physostigmine. ACh and choline were then extracted into 3-heptanone containing tetraphenylboron (5 mg ml^{-1}), back-extracted into 0.5 N HCl, and dried. For the assay of choline, the samples were incubated with choline kinase and ^{32}P-adenosine triphosphate (ATP), resulting in the formation of radioactive choline

phosphate. For the assay of ACh, the choline present in the samples was phosphorylated using non-radioactive ATP, effectively excluding > 99.5% of the endogneous choline from the ACh assay. The ACh was then hydrolyzed by acetylcholinesterase, and finally the resultant choline was phosphorylated using ^{32}P-ATP. Specific details were as previously described (Briggs *et al.*, 1985b).

Standards of authentic ACh and choline were dissolved in a portion of the same bathing solution used in each experiment, and carried through the extraction and assay with the samples. All of the drugs used in these experiments were tested for potential interference with the assay.

Quantification of the induced potentiations

Control postganglionic responses were measured over a period of 30 min or more immediately before each conditioning by preganglionic tetani or drug application. These controls were projected by least-squares regression analysis. The degree of potentiation I(t) was computed as a fractional increase in the postganglionic response at time t after the conditioning:

$$I(t) = (V_t - V_c)/V_c \quad (1)$$

where V_t is the amplitude of the postganglionic response at time t and V_c is the projected control postganglionic response.

However, a different computation was used when the release of ACh was also measured because of the smaller number of samples. ACh release measured 15–45 min after tetani was averaged and compared to the average control ACh release measured 0–30 min before the tetani in normal Ca^{2+}, or before the transition to low Ca^{2+}. Similarly, the average postganglionic response measured 27–32 min after tetani was compared to the average control response measured 0–5 min before the tetani in normal Ca^{2+}, or before the transition to low Ca^{2+}.

The evoked release of ACh was computed by subtracting the spontaneous release, determined at the beginning of each experiment, from the total release during preganglionic stimulation. Control experiments demonstrated that the spontaneous release of ACh was not altered 10 min or more after preganglionic tetani (Briggs *et al.*, 1985b), nor by the drugs that altered ACh release during stimulation: isoprenaline, 2-chloroadenosine, forskolin, and 8-bromo-cyclic AMP.

Effects of drugs on the induced potentiations

A 'bracketing' protocol was used to determine the effects of phosphodiesterase and adenylate cyclase inhibitors on the potentiations induced by tetani, forskolin, and the exogenous cyclic nucleotides. For example, l.t.p. was sequentially induced at least three times within the same ganglion: first, in the absence of any phosphodiesterase or adenylate cyclase inhibitor; next, after exposure to one of these drugs for 60–120 min; and, finally, after washing out the drug for 90–180 min. In each ganglion, the l.t.p. induced in the presence of the test drug was compared to the average of the l.t.p. induced before exposure to the drug and the l.t.p. induced after wash-out of the drug.

Data are presented as mean ± s.e.mean with the number of experiments indicated in parentheses.

Materials

Receptor agonists and antagonists Atropine sulphate, 2-chloroadenosine (6-amino-2-chloropurine riboside), hexamethonium bromide, (−)-isoprenaline hydrochloride, and (+)-tubocurarine chloride were from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

Acetylcholine release and assay Acetylcholine bromide, acetylcholinesterase (Type V, lyophilized salt-free, from electric eel), choline kinase, and physostigmine sulphate were from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Choline chloride was from Matheson, Coleman, and Bell Manufacturing Chemists, Norwood, Ohio, U.S.A. [γ-^{32}P]-ATP (6000 Ci mmol^{-1}) was from New England Nuclear, Boston, Massachusetts, U.S.A.

Nucleotides and forskolin 8-Bromo-AMP (8-bromoadenosine 5′-monophosphate, free acid), 8-bromo-cyclic AMP (8-bromoadenosine 3′:5′-cyclic monophosphate, sodium salt), 8-bromo-cyclic GMP (8-bromoguanosine 3′:5′-cyclic monophosphate, sodium salt), 8-bromo-cyclic IMP (8-bromoinosine 3′:5′-cyclic monophosphate, free acid), dibutyryl-cyclic AMP (N^6, $O^{2'}$-dibutyryladenosine 3′:5′cyclic monophosphate, sodium salt), dibutyryl-cyclic GMP (N^2, $O^{2'}$-dibutyrylguanosine 3′:5′cyclic monophosphate, sodium salt), monobutyryl-cyclic AMP (N^6-monobutyryladenosine 3′:5′cyclic monophosphate, sodium salt), monobutyryl-cyclic GMP (N^2-monobutyrylguanosine 3′:5′cyclic monophosphate, sodium salt) were from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Forskolin (7-β-acetoxy-8,13-epoxy-1-α, 6-β,9-α-trihydroxy-labd-14-ene-11-one) was from Calbiochem-Behring, La Jolla, California, U.S.A.

Phosphodiesterase and adenylate cyclase inhibitors AH 21-132 (*cis*-6-(*p*-acetamidophenyl)-1,2,3,4,4a,10-b-hexahydro-8,9-dimethoxy-2-methylbenzo[c] [1,6] naphthyridin-bis(hydrogenmaleinate)) was a gift from Sandoz Ltd, Pharmaceutical Division, Research & Development, CH-4002 Basle, Switzerland. Papaverine hydrochloride was from Sigma Chemical

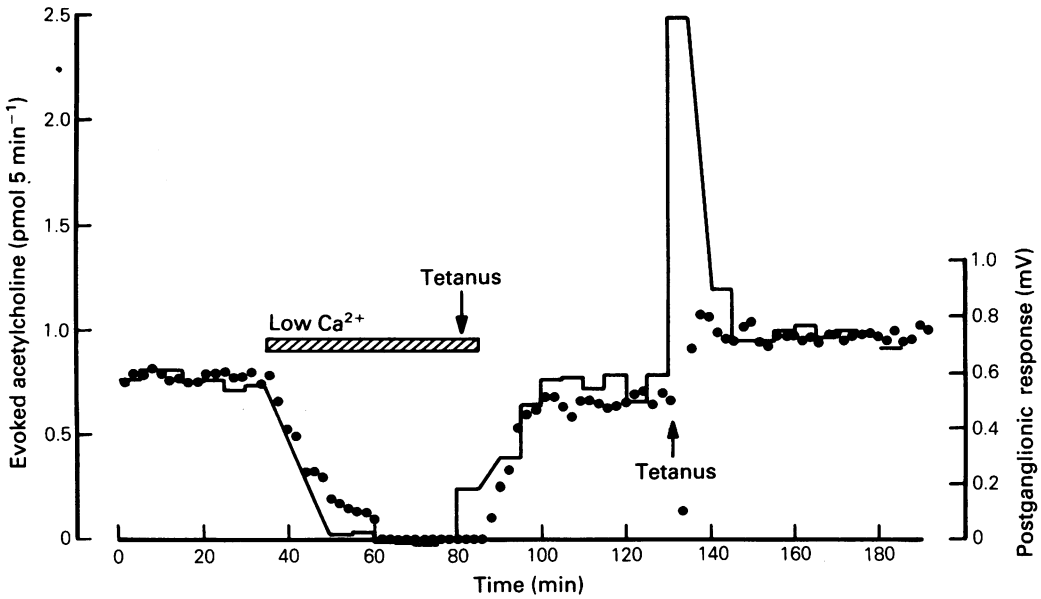

Figure 1 Calcium dependence of the long-term potentiations of acetylcholine (ACh) release and nicotinic transmission. ACh release (solid line) and the postganglionic compound action potential response (●) were measured simultaneously during 0.2 Hz stimulation. The preganglionic nerve was tetanically stimulated twice (20 Hz for 20 s, arrows). The first tetanus was in the presence of 0 mM Ca^{2+} and 8 mM Mg^{2+} to inhibit Ca^{2+} influx (hatched bar). The second tetanus was in the presence of normal Ca^{2+} and Mg^{2+} (2.2 mM and 1.2 mM). A large amount of ACh was released during the tetanic stimulation in normal Ca^{2+}, and this ACh washed out of the ganglion in the succeeding 10 min. Nicotinic transmission was inhibited briefly during this period, in part probably because of nicotinic receptor densensitization and its recovery.

Company, St. Louis, Missouri, U.S.A. SQ 20-006 (1-ethyl-4-hydrazino-1*H*-pyrazolo[3,4-b]pyridine-5-carboxylic acid, ethyl ester, hydrochloride) and SQ 22-536 (9-(tetrahydro-2-furyl)-adenine) were gifts from E.R. Squibb & Sons Inc., Squibb Institute for Medical Research, Princeton, New Jersey, U.S.A. Theophylline was from Calbiochem-Behring, La Jolla, California, U.S.A.

Results

Because the superior cervical sympathetic ganglion is rich in acetylcholinesterase, an inhibitor of this enzyme, physostigmine, was routinely added to the bathing media for the measurement of ACh release. The spontaneous release of ACh was 0.15 ± 0.02 pmol 5 min^{-1}, while the total release of ACh during 0.2 Hz preganglionic stimulation was 1.0 ± 0.07 pmol 5 min^{-1} (mean ± s.e.mean, $n = 24$). The spontaneous release of choline was 7.2 ± 0.8 pmol 5 min^{-1} ($n = 16$); this could have contributed 0.03 pmol 5 min^{-1} or less to the ACh measurement.

In the absence of an anticholinesterase, we could not detect any ACh escaping the ganglion, even after high-frequency stimulation (20 Hz for 20 s). The efflux of choline did appear to be increased by preganglionic stimulation under these conditions, probably as the result of hydrolysis of the released ACh. However, this evoked release could not be quantified satisfactorily because of the larger spontaneous release of choline.

Neurogenic long-term potentiation

The long-term potentiation (l.t.p.) of evoked ACh release was induced in a Ca^{2+}-dependent manner. Tetanic preganglionic stimulation (20 Hz for 20 s) in low Ca^{2+} did not induce a potentiation of the evoked release of ACh or the postganglionic response exceeding 15% ($n = 5$). When a normal concentration of Ca^{2+} was present during subsequent tetanic stimulation, there was an induction of l.t.p. and a concomitant increase in ACh release ($n = 4$; see Figure 1).

After preganglionic tetani in low Ca^{2+}, evoked ACh release in normal Ca^{2+} was 105 ± 7% of control and the postganglionic response amplitude was 99 ± 8% of control ($n = 4$). After tetanic stimulation of the same ganglia in normal Ca^{2+}, evoked ACh release was potentiated to 133 ± 6% of control, and the postgan-

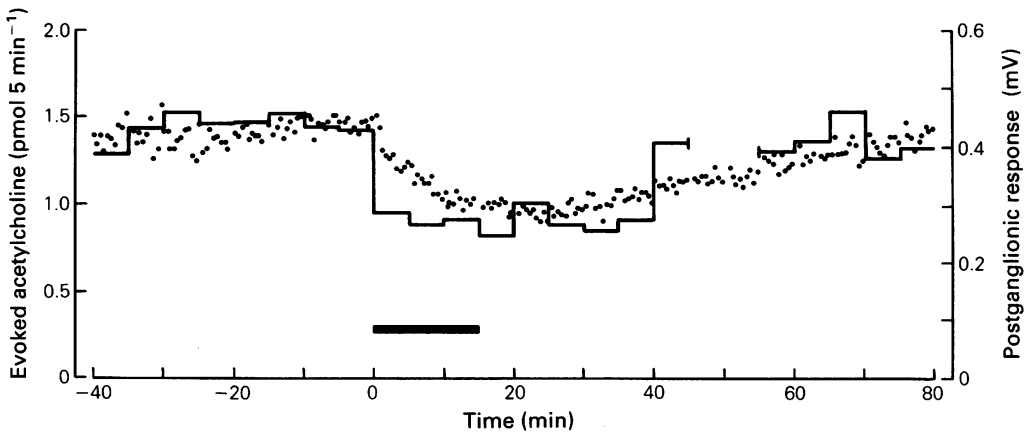

Figure 2 Inhibition of endogenous acetylcholine (ACh) release and nicotinic transmission by an adenosine receptor agonist. 2-Chloroadenosine (3 μM, solid bar) was applied for 15 min while ACh release (solid line) and the postganglionic compound action potential (●) were measured simultaneously during continuous 0.2 Hz preganglionic stimulation. Both ACh release and the postganglionic response were reversibly inhibited by the adenosine analogue.

glionic response was potentiated to 149 ± 3% of control.

Receptor-mediated regulation

The β-adrenoceptor agonist, isoprenaline (1–3 μM for 5–10 min), potentiated both the postganglionic response, by 17 ± 3%, and the evoked release of endogenous ACh, by 24 ± 2% ($n = 3$). These effects were measured simultaneously during preganglionic stimulation at 0.2 Hz. The effect of isoprenaline was sensitive to the rate of test stimulation. In individual ganglia, the potentiation of the postganglionic response by isoprenaline was halved by increasing the rate of test stimulation from 0.017 Hz to 0.2 Hz (not shown).

In contrast to β-adrenoceptor agonists, adenosine receptor agonists inhibit synaptic transmission. This also appears to be due to a presynaptic mechanism. As demonstrated in Figure 2, 2-chloroadenosine (3 μM) inhibited both the evoked release of ACh, by 39 ± 5%, and the postganglionic response, by 34 ± 4% ($n = 3$).

Regulation by cyclic AMP

Both preganglionic tetani and isoprenaline increase cyclic AMP levels in the ganglion (Briggs *et al.*, 1982; Volle *et al.*, 1982), potentiate nicotinic transmission, and potentiate evoked ACh release. We therefore tested the hypothesis that cyclic AMP mediates l.t.p. Figure 3 demonstrates that forskolin, which activates the synthesis of cyclic AMP by adenylate cyclase (Daly, 1984), does indeed cause a potentiation of both the postganglionic response and the evoked release of endogenous ACh. These potentiations developed slowly, reaching a peak 15 min or more after a 3 min application of the drug, and persisted for more than an hour. In five experiments, 1 μM to 100 μM forskolin applied for 5 min potentiated the postganglionic response by 16% to 41%, and the evoked release of ACh by 37% to 66%. The spontaneous release of ACh was not increased by forskolin (1 to 100 μM).

Another approach was to expose the tissue to exogenous cyclic AMP derivatives that are likely to penetrate the plasma membrane and activate intracellular cyclic AMP-dependent processes. The analogue 8-bromo-cyclic AMP (1 mM for 10 min) also potentiated both the postganglionic response, by 31%, and the evoked release of ACh, by 33% ($n = 2$). The spontaneous release of ACh was not increased by 8-bromo-cyclic AMP. The butyryl derivatives of cyclic AMP (1 mM for 3 min) also induced a long-lasting potentiation of the postganglionic response (Table 1). The most effective derivative was 8-bromo-cyclic AMP and the least effective was monobutyryl-cyclic AMP. However, authentic cyclic AMP (1 mM for 3 min) did not cause a potentiation of the postganglionic response, possibly because it is catabolized rapidly or is relatively impermeant.

Specificity of the cyclic nucleotide analogues

While dibutyryl-cyclic AMP (1 mM for 3 min) caused a long-lasting potentiation of transmission, treatment of the same ganglia with dibutyryl-cyclic GMP produced little or no effect ($n = 6$; see Figure 4). Similarly, monobutyryl-cyclic AMP induced a long-lasting potentiation but monobutyryl-cyclic GMP did not

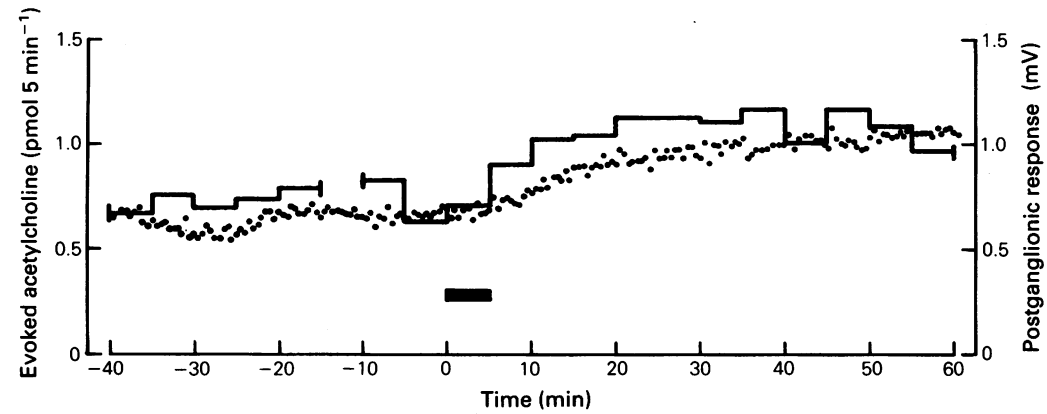

Figure 3 Forskolin-induced potentiation of evoked acetylcholine release and nicotinic transmission. Forskolin (10 μM, solid bar) was applied for 5 min during continuous preganglionic stimulation at 0.2 Hz. This induced a slowly-developing potentiation of both ACh release (solid line) and the postganglionic response (●) measured simultaneously in the same ganglion.

Table 1 Potentiation of nicotinic transmission by cyclic nucleotide derivatives

	(n)	*Peak potentiation* *I(t)*	*t* (min)
Monobutyryl-cyclic AMP	(4)	0.12 ± .01	35 ± 5
Dibutyryl-cyclic AMP	(6)	0.28 ± .03	36 ± 4
8-Bromo-cyclic AMP	(24)	0.40 ± .04	18 ± 1
8-Bromo-cyclic GMP	(12)	0.49 ± .06	51 ± 3
8-Bromo-cyclic IMP	(6)	0.18 ± .04	24 ± 3

A potentiation of the postganglionic response amplitude was induced by transient application of each cyclic nucleotide analogue (1 mM) for 3 min. Data (mean ± s.e.mean, *n* experiments) show the fractional increase of the postganglionic response at the peak of the potentiation (I(t); eqn. (1) Methods) and the time (t) at which the peak effect occurred relative to the end of the cyclic nucleotide application.

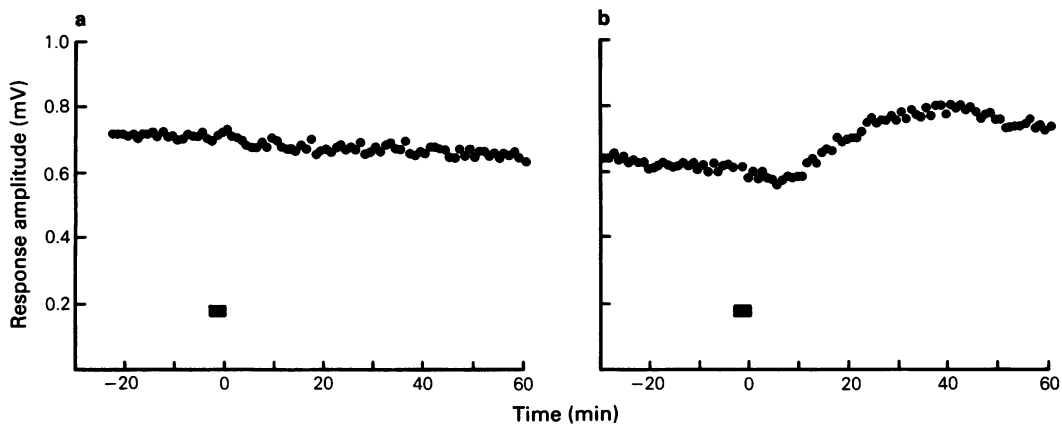

Figure 4 Specificity of the dibutyryl cyclic nucleotide derivatives. The postganglionic response was measured during continuous preganglionic stimulation at 0.017 Hz. (a) Application of dibutyryl-cyclic GMP (1 mM for 3 min, solid bar) had little or no effect on the postganglionic response. However, (b) subsequent application of dibutyryl-cyclic AMP (1 mM for 3 min, solid bar) induced a long-lasting potentiation of the postganglionic response. The data are from one ganglion.

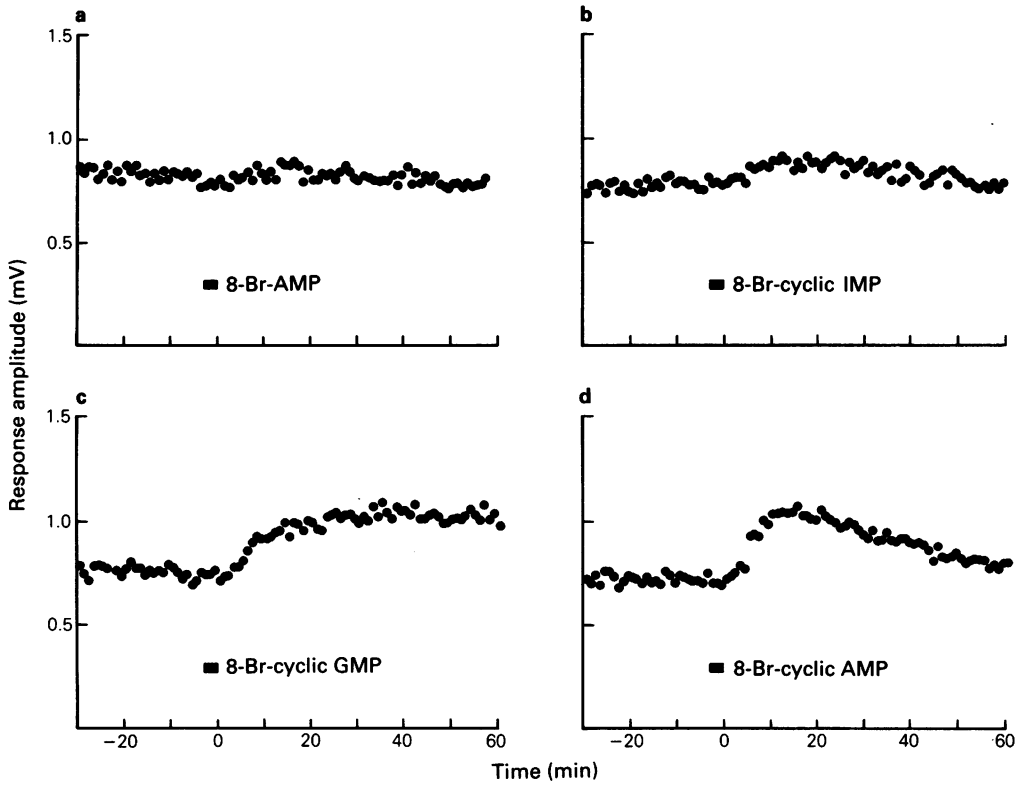

Figure 5 Specificity of the 8-bromo nucleotide derivatives. The postganglionic response was measured during continuous preganglionic stimulation at 0.017 Hz. Application of 8-bromo-AMP had little or no effect on the postganglionic response (a). However, 8-bromo-cyclic IMP caused a small potentiation (b), 8-bromo-cyclic AMP caused a larger potentiation (c), and 8-bromo-cyclic GMP caused a long-lasting potentiation of the post-ganglionic response (d). The derivatives (1 mM) were applied for 3 min each in the following order: 8-bromo-AMP; 8-bromo-cyclic IMP; 8-bromo-cyclic GMP; 8-bromo-cyclic AMP. The longer-lasting potentiation induced by 8-bromo-cyclic GMP was due to the action of the drug and not the order of application, as demonstrated by other experiments with the cyclic AMP and cyclic GMP derivatives. All data in this figure are from one ganglion.

($n = 6$; not shown). This suggests a strong specificity for the cyclic AMP analogue.

When the 8-bromo derivatives were tested, there was less apparent specificity for cyclic AMP. One such experiment is shown in Figure 5, with the replications summarized in Table 1. While 8-bromo-AMP (1 mM for 3 min) had little or no effect on the postganglionic response ($n = 8$), three different cyclic nucleotides did elicit a long-lasting potentiation of transmission. Indeed, the peak potentiation in response to 8-bromo-cyclic GMP was $104 \pm 9\%$ of that in response to 8-bromo-cyclic AMP when these substances were compared in the same ganglia ($n = 12$), and the duration of the response to 8-bromo-cyclic GMP was even longer than that to 8-bromo-cyclic AMP. The potentiation induced by 8-bromo-cyclic AMP decayed with an exponential time constant of 90 ± 16 min ($n = 23$) while that for 8-bromo-cyclic GMP was 1700 $\pm$ 800 min ($n = 12$). In response to 8-bromo-cyclic IMP, the peak potentiation was $57 \pm 15\%$ of that to 8-bromo-cyclic AMP in the same ganglia ($n = 6$); the durations of these potentiations were similar.

Interestingly, the potentiations caused by the cyclic nucleotide derivatives developed slowly, reaching a peak 15 min or more after terminating the 3 min application (Table 1).

Theophylline augmented the potentiation induced by 8-bromo-cyclic AMP, but in a manner that appeared to correlate with blockade of adenosine receptors rather than with inhibition of phosphodiesterase (Table 2). Theophylline was as efficacious at low concentrations (10–30 μM), where blockade of adenosine receptors is the primary effect, as at high concentrations (1 mM), where inhibition of phosphodiesterase becomes appreciable (Wolff *et al.*, 1981). Additionally, the cyclic AMP derivatives

Table 2 Effect of theophylline on the potentiation induced by 8-bromo-cyclic AMP

	(n)	*Peak potentiation* *Control* $I(t)_c$	*In theophylline* *% of control*
10 μM Theophylline	(3)	0.63 ± 0.03	136 ± 11%
30 μM Theophylline	(6)	0.38 ± 0.02	154 ± 8%**
100 μM Theophylline	(3)	0.63 ± 0.03	177 ± 22%
1000 μM Theophylline	(5)	0.62 ± 0.04	142 ± 10%*

In each ganglion, a potentiation of the postganglionic response was induced by transient application of 8-bromo-cyclic AMP (1 mM for 3–5 min) in the absence and presence of theophylline (see Methods). I(t) is defined in Table 1. The effect of theophylline (% of control) was computed in each experiment by dividing the peak potentiation determined in the presence of theophylline ($I(t)_T$) by the peak potentiation determined in the absence of theophylline ($I(t)_c$). Data are shown as mean ± s.e.mean and significance was tested by Student's two-tailed *t* test.
*$P < 0.05$; **$P < 0.01$ versus 100%.

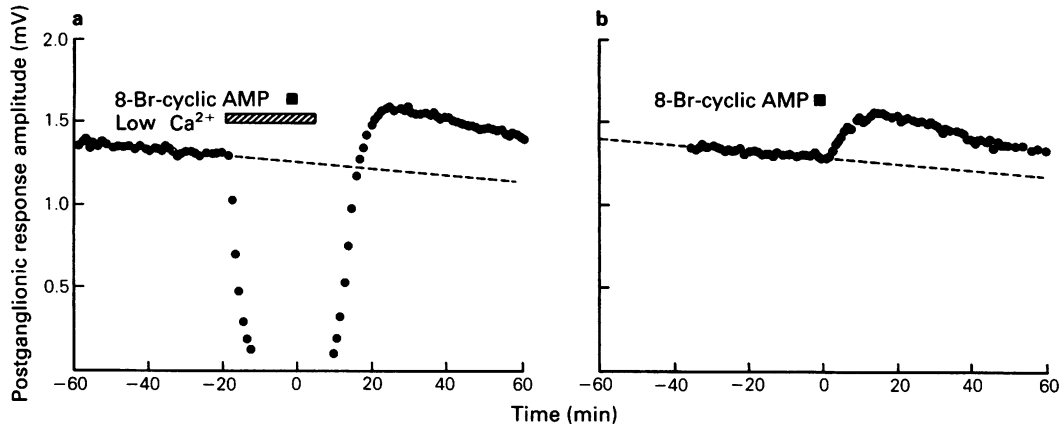

Figure 6 Calcium-independent action of a cyclic AMP analogue. The postganglionic compound action potential was measured during continuous preganglionic stimulation at 0.017 Hz. (a) Superfusion was switched to a solution containing 0 mM Ca^{2+} and 8 mM Mg^{2+} (low Ca^{2+}) for a total of 25 min. This blocked transmission. After 17 min superfusion with this low Ca^{2+}, 8-bromo-cyclic AMP (1 mM) was added for 3 min, and then washed out with the low Ca^{2+} solution for another 5 min. Finally, superfusion was returned to normal Ca^{2+} and Mg^{2+} (2.2 mM and 1.2 mM). This treatment clearly induced a potentiation of the postganglionic response relative to the control responses projected by least-squares linear regression analysis (broken line). Similar treatment with low Ca^{2+} but without 8-bromo-cyclic AMP had little or no effect (<10% apparent potentiation). (b) For comparison, 8-bromo-cyclic AMP (1 mM, 3 min) was applied subsequently in normal Ca^{2+} and Mg^{2+}. The data are from one ganglion.

sometimes elicited a small, transient inhibition of transmission (10%–15%; not shown) that preceded the potentiation and was blocked by low concentrations of theophylline (10–30 μM). Thus, the net potentiation induced by cyclic AMP derivatives may have been reduced by a smaller inhibition of transmission that was due to a non-specific activation of adenosine receptors (see also Henon & McAfee, 1983b). Under other conditions, the balance between non-specific activation of adenosine receptors and activation of cyclic AMP-dependent processes could differ, perhaps causing inconsistent results.

The potentiation induced by 8-bromo-cyclic AMP (1 mM for 3 min) was not diminished when the analogue was applied in a low Ca^{2+}, high Mg^{2+} solution that blocked synaptic transmission ($n = 4$; see Figure 6). Similarly, the actions of 8-bromo-cyclic GMP (1 mM for 3 min, $n = 4$) and of forskolin (10 μM for 3 min, $n = 4$) did not appear to be dependent upon extracellular Ca^{2+} (not shown).

Table 3 Forskolin-induced potentiation: effect of phosphodiesterase and adenylate cyclase inhibitors

Drug	(n)	Peak potentiation: Control $I(t)_c$	Peak potentiation: In drug % of control
None	(6)	0.67 ± .05	104 ± 6%
Adenylate cyclase inhibitor			
SQ 22-536, 100 μM	(2)	0.80	236%
Phosphodiesterase inhibitors			
AH 21-132, 30 μM	(6)	0.31 ± .03	160 ± 5%***
100 μM	(2)	0.41	320%
Papaverine, 5 μM	(4)	0.25 ± .02	160 ± 12%*
20 μM	(2)	0.73	290%
SQ 20-006, 100 μM	(6)	0.52 ± .04	170 ± 12%**
Theophylline, 1 mM	(6)	0.39 ± .11	112 ± 19%

Forskolin (1–3 μM for 3 min) was used to induce a potentiation of the postganglionic response in the absence and then in the presence of phosphodiesterase and adenylate cyclase inhibitors. In some experiments, ganglia were not exposed to a phosphodiesterase or adenylate cyclase inhibitor, but the protocol was otherwise similar (Drug: None). This tested the reproducibility of the forskolin-induced potentiation. Data were computed as in Table 2.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus 100%.

Effects of phosphodiesterase inhibitors

Phosphodiesterase inhibitors inhibit the hydrolysis of cyclic AMP, and thus might be expected to augment processes mediated by cyclic AMP.

The forskolin-induced potentiation of synaptic transmission was augmented by 60–70% in the presence of AH 21-132 (30 μM), papaverine (5 μM), or SQ 20-006 (100 μM) as shown in Table 3. Higher concentrations of AH 32-132 (100 μM) and papaverine (20 μM) augmented the forskolin-induced potentiation even more, but also reduced the control postganglionic response by 40% and 60% respectively. Theophylline itself increased the postganglionic response by 10–30%, but did not consistently augment the effect of forskolin.

The magnitude of neurogenic l.t.p. at 30 min and 60 min post-tetani is shown in Table 4. L.t.p., like the forskolin-induced potentiation, was consistently augmented by SQ 20-006 at both 30 and 60 min post-

Table 4 Neurogenic long-term potentiation (l.t.p.): effect of phosphodiesterase and adenylate cyclase inhibitors

Drug	(n)	30 min Post-tetani: Control $I(30)_c$	30 min Post-tetani: In drug % of control	60 min Post-tetani: Control $I(60)_c$	60 min Post-tetani: In drug % of control
None	(6)	0.19 ± .02	88 ± 8%	0.15 ± .02	92 ± 15%
Adenylate cyclase inhibitor					
SQ 22-536, 100 μM	(2)	0.20	126%	0.14	166%
Phosphodiesterase inhibitors					
AH 21-132, 30 μM	(6)	0.34 ± .01	109 ± 8%	0.26 ± .01	106 ± 15%
Papaverine, 5 μM	(6)	0.14 ± .01	110 ± 7%	0.10 ± .01	151 ± 18%*
SQ 20-006, 100 μM	(5)	0.18 ± .01	135 ± 10%*	0.14 ± .01	160 ± 20%*
Theophylline, 1 mM	(6)	0.23 ± .04	97 ± 26%	0.16 ± .04	194 ± 121%

L.t.p. was induced by brief preganglionic tetanic stimulation (5 Hz for 5 s), and the potentiation of the postganglionic response was measured as a fractional increase (I(t)) at t = 30 and t = 60 min after tetani. Otherwise, the protocol was similar to that in which forskolin was used to induce a potentiation of transmission (Table 3). Data were computed and are shown similarly.
*$P < 0.05$ versus 100%.

tetani. Papaverine augmented l.t.p. at 60 min but not at 30 min post-tetani, apparently reflecting a prolongation of l.t.p. rather than an augmentation of its magnitude. AH 21-132 did not augment the magnitude or the duration of l.t.p. Thus, the phosphodiesterase inhibitors had varied effects on neurogenic l.t.p., and one phosphodiesterase inhibitor (AH 21-132) did not alter l.t.p. but did augment the effect of forskolin.

Unfortunately, the phosphodiesterase inhibitors also had a non-specific effect that reduced the control postganglionic response. The drugs were used at concentrations that reduced the control postganglionic response by 20% or less, but these concentrations were not maximally-effective for phosphodiesterase inhibition.

Effect of an adenylate cyclase inhibitor

Adenylate cyclase inhibitors should block the synthesis of cyclic AMP, and thereby block the initiation of processes that are mediated by cyclic AMP. The adenylate cyclase inhibitor SQ 22-536 has been found to inhibit the isoprenaline-induced increase in cyclic AMP levels by 94% (Brown & Dunn, 1983b). However, in our hands SQ 22-536 (100 μM) did not inhibit the forskolin-induced potentiation of nicotinic transmission (Table 3). If anything, forskolin appeared to be more effective in the presence of SQ 22-536 than in its absence. Neurogenic l.t.p. likewise was not inhibited by SQ 22-536 (Table 4).

Discussion

Locus of long-term potentation

Brief preganglionic tetani induce a l.t.p. of nicotinic transmission and of evoked ACh release (Briggs *et al.*, 1985b). Furthermore, both potentiations were induced in a Ca^{2+}-dependent manner. This additional correlation strengthens the idea that nicotinic l.t.p. is due, at least in part, to a potentiation of synaptic ACh release.

Other studies in a variety of tissues also suggest that l.t.p. is due to a potentiation of neurotransmitter release (Baxter *et al.*, 1985; Koyano *et al.*, 1985; Lynch *et al.*, 1985; Walters & Byrne, 1985; Desmond & Levy, 1986; Feasey *et al.*, 1986; Goelet *et al.*, 1986; Applegate *et al.*, 1987). However, others suggest that postsynaptic processes explain or contribute to the expression of l.t.p. (Lynch & Baudry, 1984; Koyano *et al.*, 1985; Kessler *et al.*, 1986; Desmond & Levy, 1987). There may be more than one type of l.t.p., even in one tissue. In sympathetic ganglia, the 'long-term enhancement' of muscarinic responses appears to be due to a postsynaptic mechanism (Mochida *et al.*, 1987; see also Weight, 1984). However, nicotinic l.t.p. is distinct from muscarinic long-term enhancement because atropine does not reduce nicotinic l.t.p. under our conditions (Briggs *et al.*, 1985a).

Receptor-mediated regulation

Adenosine and adenine nucleotides may be released from the ganglion during stimulation (McCaman & McAfee, 1986), but we find no evidence that adenosine mediates l.t.p. Adenosine generally inhibits synaptic transmission, and appears to act by a presynaptic mechanism (review: Phillis & Barraco, 1985). Our studies extend that idea to sympathetic ganglia, demonstrating a simultaneous inhibition of evoked ACh release and nicotinic transmission by 2-chloroadenosine. Furthermore, low concentrations of theophylline (30 μM), sufficient to block adenosine receptors, did not alter the formation of nicotinic l.t.p. (data not shown).

β-Adrenoceptor agonists, on the other hand, can effect a long-lasting potentiation of nicotinic transmission. While endogenous noradrenaline does not appear to be responsible for the l.t.p. induced by brief preganglionic tetani (Briggs *et al.*, 1985a), hormonal adrenaline may regulate nicotinic transmission. The β-adrenoceptor agonist isoprenaline, like preganglionic tetani, was found to potentiate both evoked ACh release and nicotinic transmission. The magnitude of the potentiation of endogenous ACh release observed by us was similar to the magnitude of the potentiation of [^{3}H]-ACh release observed by Brown & Dunn (1983a). However, we find that this potentiation is sensitive to the rate of test stimulation, being half as great at 0.2 Hz as at 0.017 Hz (1 min^{-1}). The reason for this inverse dependence upon stimulation frequency is unknown, but may be related to the release of endogenous noradrenaline or other substances (see also Araujo & Collier, 1986).

The idea that cyclic AMP may mediate the β-adrenoceptor-induced potentiation of nicotinic transmission is prompted by several findings: (1) β-adrenoceptor agonists stimulate cyclic AMP synthesis potently and efficaciously in the whole sympathetic ganglion (Briggs *et al.*, 1982; Volle *et al.*, 1982); (2) preganglionic denervation reduces the amount of cyclic AMP formed following exposure to β-adrenoceptor agonists (Quenzer *et al.*, 1980); (3) β-adrenoceptor agonists and cyclic AMP both increase the quantal content of nicotinic transmission in the frog sympathetic ganglion (Kuba & Kumamoto, 1986), and both increase nicotinic transmission and evoked ACh release in the rat sympathetic ganglion (this study). However, other evidence indicates that β-adrenoceptor agonists may also act postsynaptically in the sympathetic ganglion (Brown & Dunn, 1983a), and that the potentiation is not mediated by cyclic

AMP (Brown & Dunn, 1983b).

Cyclic AMP and long-term potentiation

While several metabolic processes have been proposed to account for l.t.p. in the hippocampus (Lynch & Baudry, 1984; Stanton & Sarvey, 1985a,b; Akers *et al.*, 1986; Malenka *et al.*, 1986), there is persuasive evidence in *Aplysia* that cyclic AMP mediates a long-lasting presynaptic potentiation through inhibition of K^+ channels (Kandel & Schwartz, 1982; Abrams *et al.*, 1984; Schuster *et al.*, 1985; Walters & Byrne, 1985; cf. Saitoh & Schwartz, 1985). In vertebrates, cyclic AMP appears to increase neurotransmitter and hormone release generally (see Reese & Cooper, 1984; Mulder & Schoffelmeer, 1985). Indeed, pharmacological elevation of cyclic AMP in the sympathetic ganglia of frog and rat was associated with an increase in the quantal content of nicotinic excitatory postsynaptic potentials (e.p.s.ps) (Akagi & Kudo, 1985; Kuba & Kumamoto, 1986).

Our experiments with forskolin and cyclic AMP analogues indicated that cyclic AMP, like preganglionic tetani, could enhance both nicotinic transmission and ACh release. The magnitudes and durations of these effects were similar to the l.t.p. induced by tetani. However, it is not known to what extent the durations were influenced by the time required to wash the drugs out of the ganglion.

The finding that 8-bromo-cyclic GMP also potentiated the postganglionic response raises some question about which cyclic nucleotide regulates transmission (see also DeFrance *et al.*, 1983). However, the butyryl derivatives of cyclic GMP did not potentiate transmission while all derivatives of cyclic AMP did potentiate transmission. Furthermore, forskolin, which has been found to stimulate cyclic AMP synthesis but not cyclic GMP synthesis (Daly, 1984), was as effective as any of the exogenous cyclic nucleotides. Thus, it seems likely that cyclic AMP can potentiate transmission physiologically. It is possible that 8-bromo-cyclic GMP acted non-specifically, perhaps through the activation of cyclic AMP-dependent protein kinase (see Walter, 1984). It is not clear why the effect of 8-bromo-cyclic GMP was longer lasting than that of 8-bromo-cyclic AMP. Two possibilities are slower catabolism of 8-bromo-cyclic GMP, or inhibition of phosphatase by 8-bromo-cyclic GMP (Walter, 1984; Nestler *et al.*, 1984).

It is apparent that cyclic AMP can potentiate nicotinic transmission by a presynaptic mechanism in the sympathetic ganglion, and that this effect could last for more than an hour. To test the idea that cyclic AMP may mediate nicotinic l.t.p. in the ganglion, we applied agents expected to alter cyclic AMP metabolism and thereby alter processes regulated by cyclic AMP. Furthermore, the effects of these agents on l.t.p. were compared to their effects on the forskolin-induced potentiation of nicotinic transmission. The forskolin-induced potentiation presumably is mediated by cyclic AMP, and, like nicotinic l.t.p., involves a potentiation of ACh release.

Blockade of adenylate cyclase would be expected to block the synthesis of cyclic AMP and thereby any response mediated by cyclic AMP. The adenylate cyclase inhibitor SQ 22-536 did not inhibit neurogenic l.t.p., but neither did it inhibit the forskolin-induced potentiation of transmission. In contrast, SQ 22-536 has been found to inhibit the β-adrenoceptor stimulation of cyclic AMP in the rat superior cervical ganglion (Brown & Dunn, 1983b) and the prostaglandin stimulation of adenylate cyclase in platelets (Harris *et al.*, 1979; 1980). The reason for this discrepancy is not clear. Perhaps SQ 22-536 is selective for certain adenylate cyclase systems, or perhaps it inhibits only the receptor-mediated activation of adenylate cyclase.

Inhibition of cyclic nucleotide phosphodiesterase would be expected to reduce the degradation of cyclic AMP and augment the amplitude or duration of a response mediated by cyclic AMP. Of four phosphodiesterase inhibitors, only theophylline did not consistently augment the forskolin-induced potentiation. Theophylline itself slightly potentiated the postganglionic response, but it is not known whether this was due to inhibition of phosphodiesterase or to some other effect of theophylline, such as inhibition of intracellular Ca^{2+} sequestration. It did not appear to be due to blockade of adenosine receptors because 30 μM theophylline, which should also block adenosine receptors, did not alter the postganglionic response during the slow test stimulation (0.017 Hz).

Of three phosphodiesterase inhibitors that did augment the forskolin-induced potentiation, only two augmented neurogenic l.t.p. One (SQ 20-006) increased the magnitude of l.t.p., while the other (papaverine) increased the duration of l.t.p. Theophylline appeared to increase the duration of l.t.p. in some but not all experiments.

The mixed results with the phosphodiesterase inhibitors do not provide strong support for the hypothesis that cyclic AMP mediates l.t.p. However, these experiments may not have tested the hypothesis critically. First, the phosphodiesterase inhibitors had to be used at submaximal concentrations, which, even so, often inhibited the postganglionic response by about 20%. These drugs might have inhibited Ca^{2+} influx or the release of a substance responsible for the formation of l.t.p. This would reduce the neurogenic induction of l.t.p. which is Ca^{2+}-dependent, but not the forskolin-induced potentiation which is not Ca^{2+}-dependent. Second, it is possible that, in the induction of neurogenic l.t.p., cyclic AMP rises abruptly at the relevant site and is then removed by catabolism and/or diffusion. Phosphodiesterase may have little effect on

the peak concentration of such a localized rise in cyclic AMP, but rather may limit the diffusion of cyclic AMP to other sites in the cell (see Hockberger & Yamane, 1987).

In summary, it appears that cyclic AMP can potentiate nicotinic transmission and ACh release in the superior cervical ganglion. Cyclic AMP may mediate l.t.p., or it may regulate the induction or maintenance of l.t.p. by increasing Ca^{2+} influx or the excitability of the nerve terminals, or it may be involved in some other regulatory process. While the available evidence does not warrant the conclusion that cyclic AMP mediates l.t.p., neither have we found any compelling evidence against that idea.

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