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ADENOSINE 3′,5′-MONOPHOSPHATE IN BOVINE SUPERIOR CERVICAL GANGLION: EFFECT OF HIGH EXTRACELLULAR POTASSIUM*

PHILIPPE ROCH and PETER KALIX
Institute of Pharmacology, University of Geneva, Geneva, Switzerland

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Abstract—The synthesis of cyclic AMP in bovine superior cervical ganglia can be activated *in vitro* by raising the K^+ content of the incubation medium. Accumulation of cyclic AMP occurs when a phosphodiesterase inhibitor is present during stimulation with high K^+. The cyclic AMP increase elicited under these conditions is rapid and roughly proportional to the extracellular K^+ concentration. Adenosine had no influence on cAMP levels in resting or stimulated ganglia. Inhibitors of the muscarinic or nicotinic action of acetylcholine did not interfere with the increase in cyclic AMP elicited by K^+. Antihistaminic agents were also without effect. Although the effect of exogenous catecholamines seemed to be additive to that of K^+ stimulation, adrenergic antagonists caused a consistent reduction of the K^+ elicited cyclic AMP accumulation; the significance of these findings is discussed. The results show that a K^+-activated cyclic AMP synthesizing system is present in superior cervical ganglion which may be partially independent from neurotransmitter activated cyclic AMP synthesis occurring in this tissue.

Among the substances that can activate the cyclic AMP synthesizing system in cerebral cortex are agents that cause depolarization of the tissue. It is thus possible to enhance the cyclic AMP synthesis in guinea pig brain slices by raising the extracellular K^+ concentration [1]. Although the cyclic AMP increases occurring during depolarization of this tissue have been repeatedly investigated [2–5], the complexity of the phenomena observed has rendered difficult a conclusive interpretation of their functional significance. The purpose of the present study was to determine the existence of such a K^+ activation of cyclic AMP synthesis in the bovine superior cervical ganglion, a much simpler structure which is part of the peripheral nervous system.

The superior cervical ganglion, whose electrophysiological (reviewed by Haefely [6]) and ultrastructural [7, 8] properties are well known, is the classical preparation of ganglionic pharmacology. Since it was found that cyclic AMP levels in this organ increase with electrical stimulation [9], treatment with cholinomimetics [10] or biogenic amines [11–14], it was of interest to investigate whether the cyclic AMP synthesis in bovine superior cervical ganglion might not also be stimulated by raising the K^+ concentration of the incubation medium. As this proved to be the case, attempts were then made to determine, by applying various agonists and antagonists, whether the increases in cyclic AMP resulting from raising the K^+ concentration and those caused by putative neurotransmitters were interdependent.

METHODS

Superior cervical ganglia (SCG) were removed from calves (3–4 months old) immediately after death and kept in an ice-cold physiological solution saturated with a gas mixture of 5% CO_2–95% O_2 and having the following composition (m-moles/l): NaCl 136; KCl 5·6; $NaHCO_3$ 20·0; NaH_2PO_4 1·2; $CaCl_2$ 2·2; $MgCl_2$ 1·2; glucose 5·5. After removal of the connective tissue, the ganglion was cut with dissecting scissors into small blocks of approximately 2 mm in each dimension. These tissue samples were equilibrated at 37° for 30 min in the solution which was gassed constantly with the CO_2/O_2 mixture. Single tissue blocks were then transferred to vials containing the incubation medium, in which part or all of the Na^+ was replaced by K^+ and to which phosphodiesterase-inhibitors, putative neurotransmitters, their antagonists, etc., were added. With the exception of some experiments appearing in Table 1, the incubation medium used for stimulation, as well as that used for the unstimulated control samples, contained 10^{-2} M theophylline. Since there was considerable variation in the cyclic AMP content of the unstimulated controls of different experiments (e.g. 10·9–44·2 pmoles cyclic AMP/mg protein for 9 min of exposure to theophylline alone, n = 62), it seemed appropriate to express the value obtained under stimulating conditions as a percentage of the average cyclic AMP content observed in unstimulated ganglia in the same experiment.

At the end of the incubation period, ganglia were dropped into ice-cold 0·2 N ethanolic HCl and homogenized. The insoluble material was then separated by low-speed centrifugation, suspended in 1 N NaOH and assayed for protein according to the method of Lowry *et al.* [15]. The supernatant was evaporated in a stream of nitrogen, the residue taken up in buffer (composition: theophylline 8 mM, 2-mercaptoethanol 6 mM, Tris–HCl 100 mM, pH 7·4) and assayed for cyclic AMP by means of the saturation binding method as described by Brown *et al.* [16]. The gift of the following substances is gratefully acknowledged—burimamide: Smith, Kline & French, Welwyn

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Garden City, U.K; mepyramine: Bayer AG, Leverkusen, Germany; propranolol (Inderal®): ICI Macclesfield, U.K; phentolamine (Regitine®): CIBA-Geigy, Basle, Switzerland; 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724): Hoffmann La-Roche, Basle, Switzerland. Other chemicals were obtained from commercial sources—L-noradrenaline bitartrate, dopamine hydrochloride: Sigma Chemicals, St. Louis, Mo, U.S.A.; theophylline, adenosine, atropine sulphate: E. Merck AG, Darmstadt, Germany.

RESULTS

A preliminary series of experiments (Table 1) indicated that the cyclic AMP content of bovine SCG tissue blocks did not change significantly when the K^+ concentration of the incubation medium was raised from 5·6 to 100 mM for a 3 min period. However, a response to high extracellular K^+ could be obtained when an inhibitor of phosphodiesterase was added to the medium. When both the control and the experimental incubation medium contained 10 mM theophylline, the cyclic AMP content in tissue exposed to high K^+ was about $1\frac{1}{2}$ times that of the unstimulated controls. Although in later experiments the stimulatory effect of potassium in the absence of theophylline (as well as when it was present) was somewhat more marked, the response remained insufficient to allow for pharmacological analysis of the cyclic AMP increase caused by potassium depolarization. Therefore, in order to inhibit the breakdown of the cyclic AMP produced during K^+-stimulation, all further experiments were performed with solutions containing theophylline. Figure 1 shows the cyclic AMP accumulation as a function of time occurring under these conditions when the ganglia were stimulated with 100 mM K^+. The increase was rapid and reached its maximum within a few minutes; a standard incubation time of 9 min was therefore chosen for subsequent experiments. When increasing amounts of sodium in the Locke solution used for incubation were replaced by potassium, there was a dose-related increase in the cyclic AMP content of the tissue (Fig. 2). The threshold concentration of potassium required for stimulation appeared to be about 20 mM; with higher concentrations the amplitude of the cyclic AMP increase was roughly proportional to the amount of K^+ present in the incubation medium.

In guinea-pig cerebral cortical slices theophylline has an *antagonistic* effect on the increase in cyclic

Table 1. Effect of potassium on the cAMP content of bovine superior cervical ganglia in the absence of presence of theophylline

Addition	pmoles cAMP/mg protein
None	14·8 ± 1·3
K^+ 100 mM	16·8 ± 2·3
Theophylline 10 mM	18·6 ± 1·5
K^+ 100 mM + theophylline 10 mM	28·6 ± 0·5

Ganglion blocks were equilibrated for 30 min in Locke solution and then incubated for 3 min in media containing the above agents. Values given are mean ± S.E.M. from 3 to 17 experiments, line 1 and 3 show data obtained in previous experiments [14].

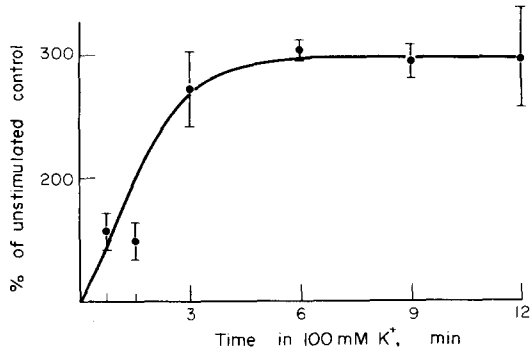

Fig. 1. Time course for the accumulation of cyclic AMP in blocks of bovine superior cervical ganglia incubated in 100 mM K^+ and 10 mM theophylline. Values (±S.E.M.) are expressed as a percentage of the mean cAMP content of ganglia incubated in a Locke solution containing 5·6 mM K^+ and 10 mM theophylline. This mean content (pmoles cAMP/mg protein) was for 3 min: 19·3 ± 1·2 (n = 13); 6 min: 22·5 ± 2·1 (n = 12); 9 min: 23·7 ± 1·0 (n = 62); and 12 min: 24·4 ± 3·1 (n = 7).

AMP elicited by depolarizing agents and high potassium [3]. This is probably due to the fact that theophylline inhibits the action of adenosine, a compound of similar structure. Adenosine has been reported to be a potent stimulant of guinea-pig cerebral cortex adenylate cyclase [17] and it has been established that the effect of depolarizing agents in the cortex is mediated by release of adenosine [5]. Results of the present study, however, show that in bovine SCG, the cyclic AMP content was not increased by incubation in 0·1 mM adenosine for 9 min (93 ± 3% of that found in untreated tissue, seven experiments). Analogous results were obtained when the comparison was made with theophylline present in both the test and the control medium (105 ± 11%, n = 5); with a phosphodiesterase inhibitor structurally unrelated to adenosine, Ro 20-1724, present in both media, the results were similar (103 ± 4%, n = 3). Moreover, in three experiments, the increases in cyclic AMP elicited in the ganglia by 100 mM potassium were not enhanced by adenosine.

Further experiments were performed in order to determine whether the release of acetylcholine, hista-

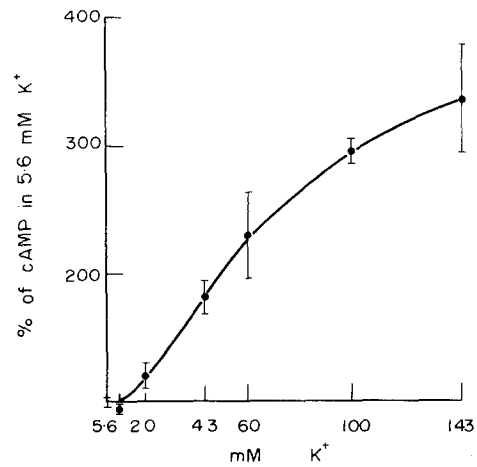

Fig. 2. Effect on the cAMP level in bovine superior cervical ganglia of 9 min exposure to media containing various concentrations of K^+ and 10 mM theophylline. Values expressed as in Fig. 1.

mine or catecholamines by depolarizing concentrations of potassium was in any way responsible for the observed increases in ganglionic cAMP. It was found that acetylcholine played no apparent part in the effect of high K^+; neither the muscarinic antagonist atropine nor the nicotinic antagonist hexamethonium reduced the increase in cyclic AMP brought about by exposure to 100 mM K^+-Locke (Fig. 3). Similarly, substances antagonizing the stimulation of an adenylate cyclase by histamine in rat [13] and bovine [14] SCG, were found to have no effect on the cyclic AMP increase caused by potassium (Fig. 3: burimamide, mepyramine).

On the other hand, when tissue samples were pre-incubated for 15 min in a medium containing substances antagonizing the effect of catecholamines on cyclic AMP levels in bovine SCG [14], the response to high potassium was inhibited. Both the alpha-antagonist phentolamine and the beta-antagonist propranolol reduced the response to 100 mM K^+ by about one third (Fig. 3). Pre-incubation in a medium containing both antagonists resulted in a 45% inhibition of the response to high potassium; a similar degree of inhibition was observed when the duration of the K^+ stimulus was reduced to 3 or 6 min. These results support the idea of a participation of endogenous catecholamines in the cyclic AMP increase elicited by high potassium media. The possibility of enhancing the synthesis of cyclic AMP in SCG by exposing the tissue to biogenic amines, particularly to catecholamines, led us to compare the effect of simultaneous stimulation with potassium and exogenous catecholamines, i.e. noradrenaline and dopamine, to that of stimulations with either agent separately. In this series of experiments it was seen (Table 2) that the increase in cyclic AMP resulting from the combination of high potassium and a catecholamine were approximately equal to those of the individual stimuli taken together.

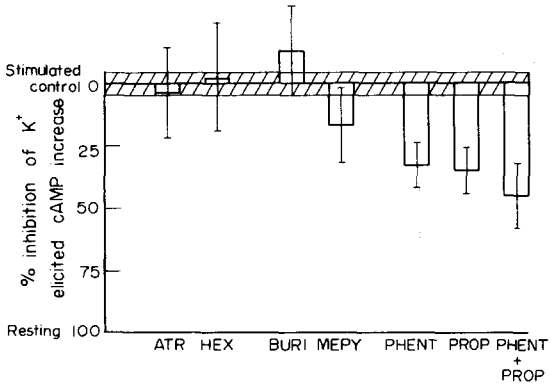

Fig. 3. Effect of anticholinergic, antihistaminic and antiadrenergic agents on the cAMP increase elicited in bovine superior cervical ganglia by 100 mM K^+ (9 min exposure) in a Locke solution containing 10 mM theophylline. The hatched bar represents the cAMP content ±S.E.M. of ganglia stimulated in the absence of antagonists, which was in this series of experiments 58·7 ± 3·6 pmoles/mg protein (n = 26). When antagonists were used they were added to the incubation medium 15 min (atropine 45 min) before and were present throughout stimulation. (ATR—atropine 0·025 mM; HEX—hexamethonium 0·500 mM; BURI—burimamide 0·100 mM; MEPY—mepyramine 0·100 mM; PHENT—phentolamine 0·100 mM; PROP—propanolol 0·100 mM; n = 3–12).

Table 2. Effect of potassium and catecholamines on the cAMP content of bovine superior cervical ganglion

Addition	cAMP content (% of control)	n
None	100 ± 5·2	18
K^+ 100 mM	264 ± 18	6
Noradrenaline 0·1 mM	196 ± 28	3
K^+ 100 mM + noradrenaline 0·1 mM	305 ± 18	4
Dopamine 0·1 mM	299 ± 27	8
K^+ 100 mM + dopamine 0·1 mM	465 ± 32	8

Ganglion blocks were equilibrated for 30 min in Locke solution and then incubated for 9 min in media containing the above agents. During the latter period 10 mM theophylline was present in both the test and the control medium. The results are expressed as a percentage (±S.E.M.) of the mean cAMP content in unstimulated ganglia which was, in this series of experiments, 23·2 ± 1·3 pmoles/mg protein (n = 18).

DISCUSSION

The results of this study demonstrate the existence of a depolarization-activated cyclic AMP synthesizing system in a structure of the peripheral nervous system, the bovine superior cervical ganglion. Exposure of this tissue to solutions with a high K^+ content causes a rapid increase in cyclic AMP levels, the magnitude of which is a function of the K^+ concentration in the incubation medium. Accumulation of cyclic AMP occurs when a phosphodiesterase inhibitor is present during stimulation with high K^+. This latter observation is analogous to that made in a previous study of catecholamine-elicited cyclic AMP increases in this same tissue [14]. It is probable that a phosphodiesterase is present in bovine SCG which limits the accumulation of cyclic AMP, whose turnover under resting conditions is relatively slow. However, the metabolism of the nucleotide is activated as soon as increased amounts of cyclic AMP are produced during stimulation. The effectiveness of this mechanism, as discussed in a previous paper [14], probably varies according to the species studied. Adenosine, with or without phosphodiesterase inhibition, was found to have no effect on the cyclic AMP level in the ganglion. Furthermore, it had no influence upon the stimulatory effect of potassium. Similarly, in rabbit SCG, adenosine was found to have no effect on cyclic AMP levels at rest or during cholinomimetic stimulation [10]. It must be assumed, therefore, that the intermediacy of adenosine is peculiar to adenylate cyclase activation in the central nervous system.

Pre-incubation with substances that interfere with the muscarinic or nicotinic action of acetylcholine did not prevent the increase in ganglionic cyclic AMP due to high extracellular potassium. Under conditions analogous to those of the present experiments, atropine is capable of blocking the increases in cyclic AMP elicited in rabbit SCG by carbachol or by electrical stimulation [10]. The effect of potassium depolarization described in this paper does not appear to involve liberation and subsequent interaction of acetylcholine with muscarinic (or nicotinic) receptors. It has been shown [13, 14] that histamine causes an increase in the concentration of cyclic AMP in SCG

and that pre-incubation with the anti-histamine burimamide can reduce this effect. However, neither burimamide nor another anti-histaminic agent, mepyramine, could reduce the response to high extracellular potassium. Thus liberation of histamine (and subsequent activation of an adenylate cyclase by this biogenic amine) does not appear to be an intermediate step in the observed effect of high K^+ media.

Catecholamine-induced cyclic AMP increases in SCG are of particular interest since it has been suggested that a dopamine-stimulated adenylate cyclase plays a modulatory role in ganglionic transmission [18]. It seemed relevant, therefore, to determine whether the effect of high K^+ could be explained in terms of release of catecholamines from intraganglionic structures. Both an alpha- and a beta-adrenergic antagonist were found to have an inhibitory effect on the response to high potassium which, on the average, seemed more pronounced when both were present simultaneously. It could therefore be concluded that catecholamines, endogenously released by the effect of high extracellular potassium, contribute substantially to the K^+-induced increase in cyclic AMP. The suggestion that the effect of high potassium is partially mediated through release is further supported by the observation that the increase in cAMP depends upon the presence of calcium ions in the incubation medium. Thus, preliminary results indicate that the response to 100 mM K^+ (9 min exposure) is reduced by $60 \pm 8\%$ ($n = 7$) when Ca^{2+} is omitted from and EGTA is added to the medium. Experiments using simultaneous adrenergic and potassium stimulation indicate, however, that the system is probably rather complex and that further experiments are needed to describe accurately the molecular mechanisms underlying the K^+ effect. It is possible, for example, that the results shown in Table 2 indicate a reciprocal potentiation between catecholamines and potassium of the kind that has been described for guinea-pig cerebral cortex [2], even though in this tissue the K^+ effect involves release of adenosine. An alternative explanation would be that some of the catecholamine containing structures in the ganglion degenerate between the time the animal is killed and potassium stimulation. K^+ would not be able to release catecholamines from such damaged structures, whereas the adjacent receptors remain sensitive to (exogenous) catecholamines.

The findings of the present study do not permit a decision as to the physiological relevance of the described phenomena. It is known that potassium ions released during nervous activity can accumulate in intercellular spaces and attain high local concentrations [19]. Interestingly, the increase of tissue cyclic AMP in rabbit SCG obtained by electrical stimulation at physiological frequencies is strongly inhibited by atropine [10], whereas the data of Fig. 3 show that the effect of high K^+ media remains unaltered when atropine is present in the medium.

It would be of interest to identify histochemically the cell type in which cyclic AMP accumulates when the extracellular potassium concentration is increased. Studies of this kind could reveal a possible interplay between the activation of cyclic AMP synthesis induced by K^+ and that induced by putative neurotransmitters.

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