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Cyclic Nucleotide Content of the Rat Anococcygeus During Relaxations Induced by Drugs or by Non-adrenergic, Non-cholinergic Field Stimulation

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Abstract—Low concentrations of sodium nitroprusside (0·2 and 1 μM) relaxed carbachol-induced tone of the rat anococcygeus but did not affect the content of either cGMP or cAMP; higher concentrations (10, 100 and 1000 μM) produced greater relaxation (> 60%) and a rise in cGMP but not cAMP. In the presence of the cGMP-phosphodiesterase inhibitor M&B 22948 (10 μM), 1 μM sodium nitroprusside produced greater relaxation and a selective increase in cGMP. Forskolin (0·5–250 μM) caused relaxation and a selective increase in cAMP; the concentration-response relationships of the two effects were similar. Non-adrenergic, non-cholinergic (NANC) field stimulation (10 Hz; 20 s trains) reduced tone by 52% but had no effect on cyclic nucleotide content; in the presence of 10 μM M&B 22948 or 1 μM sodium nitroprusside, NANC stimulation produced a greater degree of relaxation and increased cGMP but not cAMP content. The results show that NANC stimulation acts like sodium nitroprusside, causing a selective increase in cGMP, and this supports the proposal that NANC transmission in the rat anococcygeus involves an endogenous nitrate; the possibility that multiple pools of cGMP exist in the anococcygeus is discussed.

There is now strong evidence that non-adrenergic, non-cholinergic (NANC) relaxations of the anococcygeus muscle result from the release of an endogenous nitrate transmitter. In both the rat (Gillespie et al 1989) and mouse (Gibson et al 1990) anococcygeus NANC relaxations are reduced by L-N^G-monomethyl-arginine (L-NMMA), which inhibits nitric oxide (NO) synthesis from L-arginine (Moncada et al 1989). Further, L-N^G-nitro-arginine (L-NOARG), which is more potent than L-NMMA as an inhibitor of NO formation in vascular tissue (Ishii et al 1990; Moore et al 1990), is also more potent than L-NMMA as an inhibitor of NANC transmission in the anococcygeus (Gibson et al 1990; Hobbs & Gibson 1990). If the NANC transmitter is indeed an endogenous nitrate, then both NANC stimulation and nitrovasodilator drugs should activate the same cellular second messenger systems to induce relaxation. It is generally believed that nitrovasodilator drugs relax smooth muscle cells by activating guanylate cyclase and hence raising cellular levels of cyclic guanosine monophosphate (cGMP; Waldman & Murad 1987), although this may not always be the case (Nakatsu & Diamond 1989). Some indirect evidence that both NANC stimulation and nitrovasodilator drugs act through cGMP in the anococcygeus has already been obtained, since responses to both stimuli were potentiated by the cGMP-phosphodiesterase inhibitor, M&B 22948 (Gibson & Mirzazadeh 1989). The object of the present study was to obtain more direct evidence for the involvement of cGMP by measuring the content of this nucleotide in the rat anococcygeus during relaxations induced by drugs and NANC stimulation; the selectivity of any changes obtained was determined by measuring the content of cyclic adenosine monophosphate (cAMP) in the same tissue extracts.

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Materials and Methods

Materials

Drugs used were: carbachol (BDH); forskolin (Sigma); guanethidine sulphate (Ciba); M&B 22948 (May & Baker); phentolamine mesylate (Ciba); sodium nitroprusside (Sigma).

Methods

Male Wistar rats, 200–400 g, were killed by stunning and cervical dislocation. The anococcygeus muscles were dissected (Gillespie 1972) and set up in 2 mL glass organ baths containing Krebs-bicarbonate buffer (mM: NaCl 118·1; KCl 4·7; $MgSO_4$ 1·0; KH_2PO_4 1·0; $CaCl_2$ 2·5; $NaHCO_3$ 25·0; glucose 11·1) which was maintained at 37°C and gassed continuously with 95% O_2-5% CO_2. A resting tension of 1 g was placed on the tissue and changes in tension recorded with a Grass FT03 force-displacement transducer attached to a Graphtec pen-recorder (WR 3101). Muscles were allowed to equilibrate for 45 min before beginning the experiment.

Field stimulation was applied by two parallel platinum electrodes running down either side of the tissue; these were attached to a Grass S48 stimulator (1 ms pulse width; 70 V). To observe NANC relaxations to field stimulation in the anococcygeus it is necessary to raise muscle tone and negate contractions due to stimulation of sympathetic nerve endings. Tone was raised with 50 μM carbachol in all cases; sympathetic responses were attenuated by including 1 μM phentolamine in the Krebs solution and pre-incubating each muscle with 30 μM guanethidine for 10 min during the 45 min equilibration period. To record responses to relaxant drugs, tone was first raised with 50 μM carbachol; when a steady, maintained rise in tone was obtained (usually within 3 min) the relaxant drug was added to the organ bath. The response was calculated as the peak % reduction of carbachol-induced tone occurring within 4 min of adding the drug; if no peak occurred, the level of relaxation at 4 min was measured.

To estimate levels of cGMP and cAMP the tissues were frozen rapidly at the peak of the relaxation response (or at 4 min) in isopentane which had previously been cooled to the temperature of liquid N_2. To facilitate rapid freezing, the organ baths were arranged such that they could be dropped away from the tissue, leaving the muscle suspended between the electrode assembly. A polypropylene scintillation vial containing 10 mL of cooled isopentane could then be brought up to envelope the tissue, thus effecting rapid freezing. The frozen tissue was then weighed in a stainless steel crucible, pre-cooled in liquid N_2, and the nucleotides extracted by thawing the tissue in 1 mL trichloroacetic acid (TCA; 6%). After centrifugation (10 000 *g*; 10 min; 4°C), the supernatant was transferred to a 10 mL stoppered glass extraction tube; the lipids and TCA were extracted 5 times with ether (water saturated; 2·5 mL each time). The residual aqueous layer was then evaporated to dryness and stored at −20°C. On the day of assay, the dried material was dissolved in 200 μL Tris-EDTA buffer (0·05 M; pH 7·5). The cyclic nucleotide content of 100 μL (cGMP) and 50 μL (cAMP) samples was then assayed using commercially available kits (Amersham; TRK 500 for cGMP; TRK 432 for cAMP). Results were expressed as pmoles nucleotide (g tissue)$^{-1}$.

Results are given as mean ± s.e.m., with a minimum of six observations in each case. Statistical analysis was by Student's *t*-test; $P < 0.05$ was considered significant.

Results

Nucleotide levels in control tissues

In muscles frozen immediately after the 45 min equilibration period the levels of cGMP and cAMP were 26·8 ± 5·3 (n = 9) and 268 ± 48 (n = 8) pmol (g tissue)$^{-1}$, respectively; these were unchanged (25·8 ± 5·8, n = 9 and 231 ± 38, n = 8; $P > 0.05$) in muscles frozen after tone had been raised with 50 μM carbachol. Consequently, in all subsequent experiments, the control values given are those obtained from tissues exposed to carbachol but not to the relevant relaxing stimulus.

The effect of sodium nitroprusside

Sodium nitroprusside (0·2–1000 μM) produced concentration-related relaxations of carbachol-induced tone (Fig. 1a); these relaxations had reached a peak by 60 s and so cyclic nucleotides were measured in tissues frozen at this time (Fig. 1b, c). The two lower concentrations of sodium nitroprusside (0·2 and 1 μM), which reduced tone by 20 and 43%, respectively, caused no change in cGMP content, but the higher concentrations (10, 100 and 1000 μM), which reduced tone by 78, 68 and 78%, respectively, resulted in significant increases in cGMP (Fig. 1b); cAMP levels were unaffected by any of the concentrations of sodium nitroprusside used (Fig. 1c). Since the lower concentrations had reduced tone without

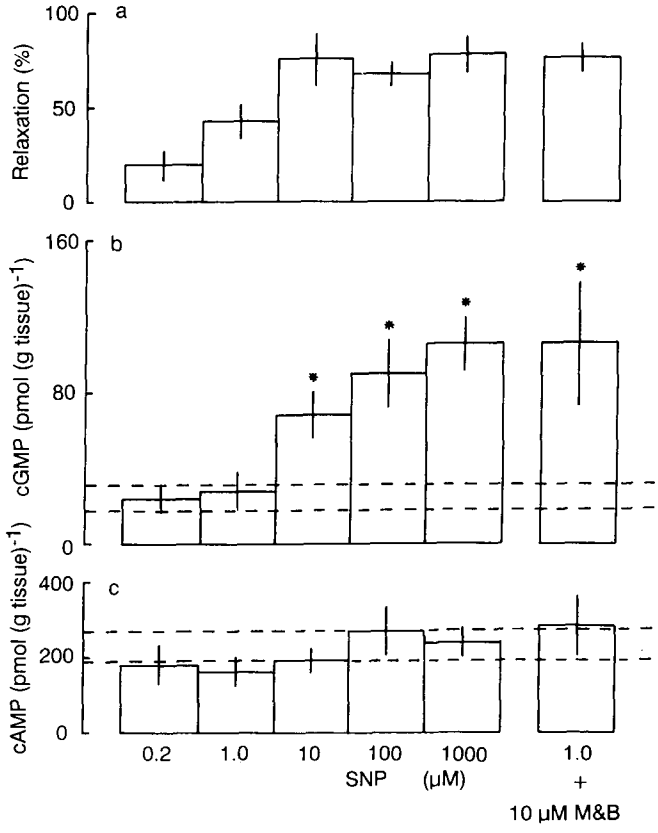

FIG. 1. Histogram showing the effect of sodium nitroprusside (SNP), alone and in combination with M&B 22948 (M&B), on relaxation of carbachol (50 μM)-induced tone (a), cGMP content (b) and cAMP content (c) of rat anococcygeus muscles. Each column represents the mean ± s.e.m. from at least six individual muscle preparations. The dotted lines give the limits of s.e.m. for cGMP and cAMP content in control tissues. The drug concentrations used are shown below each set of columns. *$P < 0.05$ compared with control.

a concomitant rise in cGMP content, we carried out some experiments using the cGMP-phoshodiesterase inhibitor M&B 22948. By itself, 10 μM M&B 22948 produced a small reduction of carbachol-induced tone (12±2%; n=12) but did not cause a change in either cGMP (18±3 pmol (g tissue)$^{-1}$) or cAMP (230±17 pmol (g tissue)$^{-1}$). However, in the presence of 10 μM M&B 22948, relaxations induced by 1 μM sodium nitroprusside were increased from 43 to 76% and there was a significant elevation of cGMP, but not cAMP (Fig. 1).

The effect of forskolin

Forskolin (0·5–250 μM), which is a known activator of adenylate cyclase (Seaman & Daley 1983), also caused concentration-related relaxations (Fig. 2a). These relaxations were much slower than those to sodium nitroprusside, but by 4 min a peak had been reached in most cases, and so muscles were frozen at this time-point. cAMP levels were elevated by forskolin (Fig. 2c) and, unlike sodium nitroprusside and cGMP, the concentration-effect relationships for forskolin-induced relaxation and cAMP elevation were similar. cGMP content was unchanged by any of the concentrations of forskolin used (Fig. 2b). In the presence of 10 μM M&B 22948, the effects of 0·5 μM forskolin on tone, cGMP and cAMP were unaffected (Fig. 2).

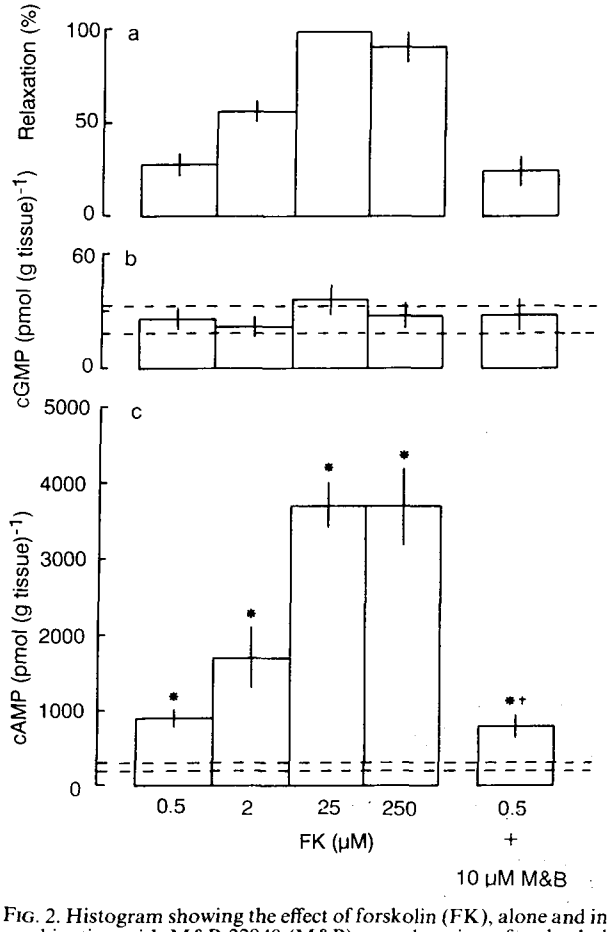

FIG. 2. Histogram showing the effect of forskolin (FK), alone and in combination with M&B 22948 (M&B), on relaxation of carbachol (50 μM)-induced tone (a), cGMP content (b), and cAMP content (c) of the rat anococcygeus muscle. Each column represents the mean±s.e.m. from at least six individual muscle preparations. The dotted lines give the limits of s.e.m. for cGMP and cAMP content in control tissues. The drug concentrations used are shown below each set of columns. *$P<0·05$ compared with control. †$P>0·05$ compared with 0·5 μM forskolin alone.

The effect of NANC stimulation

Field stimulation (10 Hz; 20 s trains) caused NANC relaxations of carbachol-induced tone. The peak effect occurred between 10 and 20 s; muscles were frozen at 20 s as this was considered to be the minimum time to allow dropping of the bath and freezing of the tissue. NANC stimulation (10 Hz) caused a relaxation of 52%, but produced no change in either cGMP or cAMP (Fig. 3).

Previous experiments had shown that sodium nitroprusside concentrations which reduced tone by >60% were required to observe increases in cGMP (Fig. 1); since NANC stimulation reduced tone by only 52% it seemed possible that it was acting like the lower concentrations of sodium nitroprusside. This possibility was tested in two series of experiments. In the first, NANC stimulation was performed in the presence of 10 μM M&B 22948; here the relaxation induced by NANC stimulation was increased to 80%, cGMP content was significantly elevated, and cAMP content was unchanged (Fig. 3). In the second series of experiments the additive effect of sodium nitroprusside and NANC stimulation was studied. Muscles were exposed to 1 μM sodium nitroprusside for 40 s and then stimulated at 10 Hz for a further 20 s before freezing. By itself, 1 μM sodium nitroprusside reduced tone by 43% but had no effect on nucleotide content (see Fig. 1); the combination of 1 μM sodium nitroprusside and 10 Hz NANC stimulation caused a relaxation of 84%, which was accompanied by a rise in cGMP, but not cAMP, content (Fig. 3). On the other hand, 0·5 μM forskolin, by itself, reduced tone by 28% and raised cAMP but not cGMP content; the combination of 0·5 μM forskolin with 10 Hz NANC stimulation caused a greater degree of relaxation (76%) but no further rise in cAMP (above that already induced by forskolin alone) and no change in cGMP content (Fig. 3).

Discussion

The results of this study have shown that relaxations of the rat anococcygeus may be associated with a rise in either cGMP or cAMP. Each nucleotide is sensitive to a specific pharmacological stimulus (sodium nitroprusside for cGMP; forskolin for cAMP) and therefore the rise in nucleotide content is not a general response secondary to, and consequent upon, relaxation. However, the pattern of response differed markedly between sodium nitroprusside and forskolin; the rise in cAMP content induced by forskolin occurs with the same concentration-effect relationship as muscle relaxation but in the case of sodium nitroprusside, low concentrations cause relaxation but no measurable change in nucleotide content. This lack of relationship between cGMP content and relaxation has been observed with nitrovasodilator drugs in other tissues (for review of subject see Nakatsu & Diamond 1989) and various hypotheses have been put forward to explain it. Most commonly, it has been suggested that cGMP may exist in multiple pools within the tissue and that the pool associated with muscle relaxation must rise markedly before an increase in the total pool, as measured in

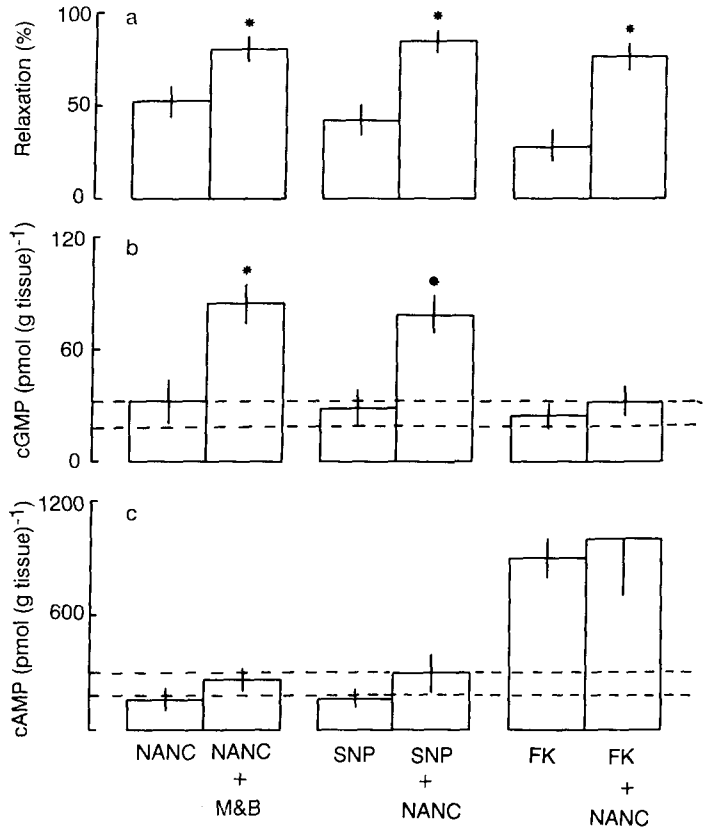

FIG. 3. The effect of non-adrenergic, non-cholinergic (NANC; 10 Hz; 20 s trains) field stimulation, alone and in combination with M&B 22948 (M&B; 10 μM), sodium nitroprusside (SNP; 1 μM) or forskolin (FK; 0·5 μM) on relaxation of carbachol (50 μM)-induced tone (a), cGMP content (b), and cAMP content (c) of rat anococcygeus muscles. Each column represents the mean ± s.e.m. from at least six individual muscle preparations. The dotted lines give the limits of s.e.m. for cGMP and cAMP content in control tissues. The various combinations used are shown below each set of columns. *$P < 0{\cdot}05$ compared with adjoining column.

the assay, is detected. In the present study, rises in cGMP were observed only when sodium nitroprusside caused relaxations of > 60%; even with the cGMP-phosphodiesterase inhibitor M&B 22948, which reduced tone by 12%, no increase in cGMP was detectable suggesting that it is not simply a high rate of turnover of cGMP which prevents increased content. However, when given in combination with M&B 22948, a low concentration (1 μM) of sodium nitroprusside produced a greater relaxation (> 60%) and a detectable increase in cGMP. Overall, these results could be explained by the existence of different pools of cGMP, and there has been recent evidence that this may be so in photoreceptor cells, in which a significant fraction of the cGMP appears to be bound to non-catalytic sites of phosphodiesterase (McNaughton 1990). However, it cannot be ruled out that some mechanism other than cGMP may contribute to the relaxant effect of sodium nitroprusside, at least at low concentrations of the nitrovasodilator (Nakatsu & Diamond 1989).

NANC stimulation, by itself, caused no change in nucleotide content. However, the relaxation induced was only 52%, the maximum effect of NANC stimulation under the conditions used in our experiments. If the multiple pool theory discussed previously is correct, then it seems possible that the maximum amount of NANC transmitter released was not sufficient to cause a large enough increase in cGMP in the sensitive pool to be detectable in the total pool. This possibility was confirmed in two ways: first, in the presence of M&B 22948 the NANC relaxation was increased and there was a detectable rise in cGMP; secondly, when the relaxant responses to NANC stimulation and 1 μM-sodium nitroprusside were combined cGMP levels were increased, even though neither stimulus by itself had such an effect. This latter response was specific for cGMP since a similar experiment carried out with forskolin and NANC stimulation produced no further increase in cGMP or cAMP. Thus, under appropriate conditions, NANC stimulation of the rat anococcygeus causes a selective rise in cGMP content; NANC stimulation also raises cGMP content in the bovine retractor penis (Bowman & Drummond 1984) and opossum lower oesophageal sphincter (Torphy et al 1986; Barnette et al 1989) suggesting that cGMP may be a second messenger substance common to several NANC neurotransmitter sites, presumably those utilizing the endogenous nitrate system.

In conclusion, the results of this study have provided direct evidence for the involvement of cGMP in NANC relaxation of the rat anococcygeus muscle but raise the possibility that either multiple pools of cGMP exist within the tissue or that some other factor may also contribute to the relaxation. The results are compatible with recent evidence that the NANC neurotransmission system in the anococcygeus involves an endogenous nitrate (Gillespie et al 1989; Ramagopal &

Leighton 1989; Gibson et al 1990; Hobbs & Gibson 1990) since both sodium nitroprusside and NANC stimulation produced similar and additive effects on muscle relaxation and nucleotide content.

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