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Influence of nitric oxide on transepithelial transport in toad skin: effects of cholinergic agents and morphine

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Abstract

The effects induced by L-arginine (L-Arg) on the short-circuit current and potential difference of *Pleurodema thaul* skin were investigated. L-Arg, but not D-Arg significantly increased the short-circuit current and potential difference when applied to the serosal surface. The effects of L-Arg were antagonized by amiloride, N^G-nitro-methyl-L-arginine (L-NAME) and by methylene blue. Carbachol and acetylcholine induced significant increases of both electrical parameters of the toad skin. These effects of the muscarinic cholinergic drugs were potentiated by a low concentration of L-Arg and antagonized by L-NAME or methylene blue. Carbachol and acetylcholine induced significant increases of both electrical parameters of the toad skin. These effects of the muscarinic cholinergic drugs were potentiated by a low concentration of L-Arg and antagonized by L-NAME or methylene blue. Addition of dibutyryl cyclic guanosyl monophosphate (db cGMP) or dibutyryl cyclic adenosine monophosphate (db cAMP) increased short-circuit current and potential difference. The effects of db cGMP, but not those of db cAMP were antagonized by L-NAME. The consecutive application of db cGMP and db cAMP induced additive effects. These results suggest that L-Arg increases transport in toad skin presumably acting through the formation of nitric oxide, which then stimulates cytoplasmic guanylate cyclase and leads to increased Na^+ and K^+ transport. The effects of L-Arg and carbachol were antagonized by acute application of morphine; however, a rebound response was observed when carbachol or noradrenaline were given after prolonged exposure of the skin to morphine, which suggests an adaptive response of the skin involving both cGMP and cAMP. Responses to both nucleotides were unchanged by morphine.

Keywords: L-Arginine; Nitric oxide (NO); Na^+ transport; K^+ transport; Opiate; Cholinergic responses

1. Introduction

A number of authors have reported the effects of acetylcholine on Na^+ transport in amphibian epithelia (McAfee, 1964; McAfee and Locke, 1967; Cuthbert and Wilson, 1981). Serosal addition of acetylcholine or carbachol to *Pleurodema thaul* skin increases the short-circuit current and potential difference denoting an increase of Na^+ transport in the preparation. The effects of cholinergic agents are inhibited by atropine suggesting the involvement of muscarinic receptors; nicotinic ligands are ineffective (Sobrevía et al., 1989).

As far as we are aware, few authors have attempted to elucidate the biochemical mechanisms involved in the cholinergic responses of epithelia. Thus, Cuthbert and Wilson (1981) measured cyclic adenosine 3′,5′-monophosphate (cAMP) content in the skin of *Rana temporaria*, and concluded that the stimulatory responses to acetylcholine or carbachol are induced through activation of adenylcyclase.

It is generally accepted that cholinergic responses in non-epithelial tissues are mediated by guanylate cyclase activation and an increase in cGMP (Murad et al., 1978; Kukovetz et al., 1979). Furchgott and Zawadzki (1980) showed that blood vessel relaxation in response to acetylcholine was mediated by endothelial release of a labile substance later identified as nitric oxide (NO). The effects of NO are inhibited by the L-arginine (L-Arg) derivative N^G-nitro-methyl-L-arginine (L-NAME). NO is considered a major regulator in the nervous, immune and cardiovascular systems (for review, see Bredt and Snyder, 1994); it is formed on demand by the action of NO synthase on L-Arg and diffuses to adjacent target cells where it activates soluble guanylate cyclase (Murad et al., 1978).

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Several studies have shown that NO modulates ion transport in epithelia. Stoos et al. (1992) found that acetylcholine and bradykinin decreased the short-circuit current and bradykinin increased the cGMP content in cultured mouse cortical collecting duct cells and this effect was partially blocked by L-NAME. Barry et al. (1994) showed that L-Arg increased ion absorption, and that L-NAME favoured ion secretion in the rabbit ileum; and the results of Rao et al. (1994) suggest that NO produces a net proabsorptive effect on ion transport in mouse ileum, which is blocked by methylene blue, an inhibitor of soluble guanylate cyclase.

Evidence from several sources has implicated the involvement of NO in the acute and chronic effects of opiates (Moore et al., 1991; Morris et al., 1992; Elliot et al., 1994). Administered acutely, opiates inhibit adenylcyclase, whereas chronic exposure leads to an upregulation of the enzyme activity, (for review, see Cox, 1993). The involvement of cGMP on morphine effects has not been as fully analyzed as the influence of cAMP. Gwynn and Costa (1982) and Muraki et al. (1984) demonstrated that opiates induce an increase of cGMP formation in cloned neuroblastoma cells and in peripheral tissues of the C57BL mouse.

This work explores: (a) the effects of L-Arg on the electrical properties of the isolated toad skin and (b) the participation of cGMP and NO in toad skin cholinoceptor activation and (c) morphine influence on the responses to carbachol, acetylcholine and noradrenaline.

2. Materials and methods

2.1. Animals and experimental procedures

Pleurodema thaul toads of either sex (16–20 g) collected in fresh water ponds in Concepción, Chile, were kept in tap water 24 h prior to use. The experiments were performed on pieces of the abdominal skin dissected from pithed toads. The skins were mounted between two halves of a Ussing perspex chamber: a circular area of 1.33 cm^2 was exposed to 3.5 ml Ringer's bathing solution on each side. The composition of the solution was (mM): Na^+ 114, K^+ 2.5, Cl^- 117.5, Ca^{2+} 2.0, HCO_3 2.3 and glucose 11. The bathing medium was stirred and oxygenated by bubbling with air. The short-circuit current was monitored with non-polarizable Ag/AgCl electrodes placed at 15 mm distance from the epithelium and connected to a voltage clamp circuit (G. Métraux Electronique) set to keep the potential difference across the skin at zero mV. The potential difference was measured with calomel-agar electrodes at intervals of 2 min for 4 s. Both parameters were monitored on a two-channel Cole-Parmer recorder. Experiments were started 30 min after the bioelectric parameters of the skin had reached a steady level.

In order to study the prolonged effects of morphine on the responses to L-Arg, cholinergic drugs and noradrenaline, the serosal surface of the skin was incubated 3 h with 10^{-5} M morphine and then carefully washed for 45 min. When the skin was stabilized, either L-Arg, acetylcholine, carbachol or noradrenaline were assayed. Control values after 3 h incubation with solvent were also examined.

2.2. Drugs

The following drugs were used in a volume sufficient to give the final concentrations mentioned in the text: L-arginine, N^G-nitro-methyl-L-arginine, acetylcholine, carbachol, noradrenaline, morphine, dibutyryl cyclic guanosine monophosphate, dibutyryl cyclic adenosine monophosphate and methylene blue, all from Sigma Chem. Co., St. Louis, MO, USA. The drugs were applied to the serosal surface, unless otherwise specified. Amiloride, a gift from Merck, Sharp and Dohme, was applied to the mucosal surface of the skin.

2.3. Statistical analysis

Throughout the text, values are expressed as means ± S.E.M. Statistical analysis was performed by means of Student's paired or unpaired *t*-test. Analysis of variance was used when applicable and the significance was calculated according to the Student-Newman-Keuls test.

3. Results

3.1. Effect of L-Arg on the electrical properties of the isolated toad skin

Responses were obtained in 53 out of 76 skins when the agent was added to the serosal surface. The concentration range used in the experiments induced a concentration-related response in potential difference and short-circuit current. (Fig. 1). Smaller concentrations were ineffective,

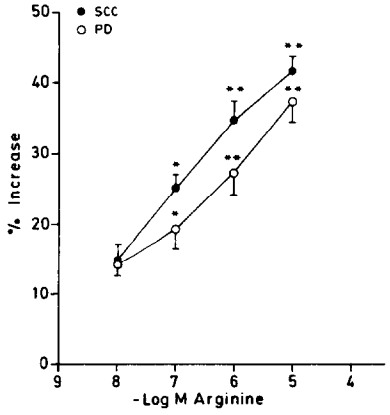

Fig. 1. The log-concentration relationship for L-Arg treated toad skins. Results are expressed as percentage increase in basal values. Each point represents mean ± S.E.M., $n = 20$. SCC, short-circuit current; PD, potential difference. Significantly different from basal values, * $P < 0.05$; ** $P < 0.01$ (Student's paired *t*-test).

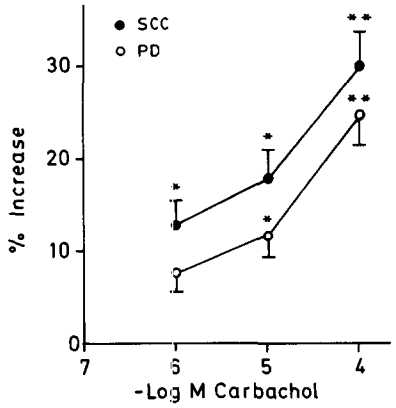

Fig. 2. The log-concentration relationship for carbachol treated toad skins. Results are expressed as percentage increase in basal values. Each point represents means ± S.E.M., $n = 14$. SCC, short-circuit current; PD, potential difference. Significantly different from basal values, * $P < 0.05$; ** $P < 0.01$ (Student's paired t-test).

and larger concentrations either had no further effect or decreased the bioelectric parameters. The time course of the response to a half maximal concentration of L-Arg was a rise to peak at 9.25 ± 0.61 min ($n = 20$) followed by a return to control level in 34.25 ± 1.50 min when the skin was not washed out. The sensitivity of the skin to L-Arg varied considerably and tachyphylaxis was a frequent problem; therefore skins were submitted to prolonged washing. L-Arg added to the mucosal surface was ineffective.

3.2. Effect of amiloride on the toad skin response to L-Arg

To find out whether L-Arg might be acting on Na^+ transport, a series of 8 experimental runs was made with 10^{-7} M L-Arg: the short-circuit current increased from 43.3 ± 3.2 μA/cm² to 54.1 ± 3.9 μA/cm² ($P < 0.01$). Ten minutes after return of the current to control level, amiloride (10^{-4} M, mucosal surface) decreased the short-circuit current from 40.0 ± 4 μA/cm² to 1.8 ± 0.08 μA/cm² and addition of L-Arg had no effect.

Table 1
Effect of L-arginine (L-Arg, 3.0×10^{-8} M) on the isolated toad skin response to acetylcholine and to carbachol

Drug	Before L-Arg		After L-Arg	
	PD (mV)	SCC (μA/cm²)	PD (mV)	SCC (μA/cm²)
Acetylcholine	20.4 ± 2.4 [a]	53.4 ± 4.2 [a]	27.2 ± 2.8 [b]	59.5 ± 3.7 [b]
10^{-6} M	(16.0 ± 2.1)	(47.4 ± 4.2)		
Carbachol	45.6 ± 2.7 [a]	50.3 ± 2.8 [a]	50.7 ± 3.8 [b]	57.4 ± 3.5 [b]
10^{-6} M	(41.3 ± 2.2)	(45.0 ± 2.5)		

Values are means ± S.E.M.; $n = 6$. PD, potential difference; SCC, short-circuit current. [a] Significantly different from basal values; [b] Significantly different from responses induced by acetylcholine or carbachol before L-Arg ($P < 0.05$, Student's paired t-test). Basal values are indicated in parentheses.

Table 2
Effect of N^G-nitro-methyl-L-arginine (L-NAME, 10^{-5} M), on the responses of isolated toad skin halves to L-arginine (L-Arg) and to carbachol

Drug	Skin half A before L-NAME		Skin half B after L-NAME	
	PD (mV)	SCC (μA/cm²)	PD (mV)	SCC (μA/cm²)
L-Arg	35.9 ± 3.1 [a]	39.8 ± 3.3 [aa]	29.2 ± 2.9 [ns]	32.1 ± 2.7 [ns]
10^{-6} M	(29.2 ± 2.7)	(30.6 ± 2.8)	(27.2 ± 1.9)	(30.0 ± 2.0)
Carbachol	34.8 ± 3.2 [a]	43.5 ± 3.5 [aa]	27.5 ± 3.0 [ns]	30.2 ± 4.1 [ns]
10^{-6} M	(27.2 ± 2.4)	(31.2 ± 3.0)	(25.2 ± 3.2)	(28.7 ± 4.5)

Values are means ± S.E.M.; $n = 6$. PD, potential difference; SCC, short-circuit current. Significantly different from control values, [a] $P < 0.05$; [aa] $P < 0.01$.; ns, not significant (Student's paired t-test). Basal values are indicated in parentheses.

3.3. Effect of L-Arg on the toad skin response to acetylcholine and carbachol

Responses to acetylcholine and carbachol (10^{-6}–10^{-4} M) were obtained for each agent in 19 out of 20 skins. The maximal concentration brought about a 24.8 ± 3.1% rise in the potential difference and a 30.5 ± 4.0% rise in the short-circuit current (Fig. 2).

These responses are similar to those induced by L-Arg and we looked for the possibility of a common mechanism of action. Table 1 shows the effects of the consecutive application of L-Arg in small concentrations (3×10^{-8} M) on the responses to both agents (10^{-6} M).

3.4. Effect of N^G-nitro-methyl-L-Arg and of methylene blue on the toad skin response to acetylcholine and to carbachol

The effects of L-NAME and of methylene blue on the toad skin responses to L-Arg and to carbachol were investigated in two sets of experimental runs, where skin half A was stimulated with L-Arg or carbachol, and skin half B was incubated 30 min in the presence of either inhibitor and then exposed to L-Arg or carbachol. Table 2 shows

Table 3
Effect of methylene blue (MB 1.0×10^{-5} M), on the responses of isolated toad skin halves to L-arginine (L-Arg) and to carbachol.

Drug	Skin half A before MB		Skin half B after MB	
	PD (mV)	SCC (μA/cm²)	PD (mV)	SCC (μA/cm²)
L-Arg	29.4 ± 2.8 [a]	36.2 ± 3.2 [a]	27.9 ± 2.7 [ns]	32.5 ± 3.7 [ns]
10^{-6} M	(24.6 ± 2.0)	(29.0 ± 3.5)	(26.2 ± 2.4)	(30.8 ± 2.7)
Carbachol	30.8 ± 3.0 [a]	36.9 ± 2.9 [aa]	20.7 ± 4.1 [ns]	28.4 ± 3.0 [ns]
10^{-6} M	(25.2 ± 3.1)	(30.1 ± 2.4)	(19.0 ± 3.5)	(26.8 ± 2.9)

Values are means ± S.E.M.; $n = 6$. PD, potential difference; SCC, short-circuit current. Significantly different from basal values, [a] $P < 0.05$; [aa] $P < 0.01$. ns, not significant (Student's paired t-test). Basal values are indicated in parentheses.

that L-Arg and carbachol applied to skin half A induced significant increases in potential difference and short-circuit current, whereas these agents applied to skin half B after exposure to L-NAME (10^{-6} M), did not induce significant changes of the bioelectric parameters. The effect of methylene blue (10^{-6} M) on L-Arg and carbachol responses is depicted in Table 3.

3.5. Effect of dibutyryl cyclic guanosyl monophosphate (db cGMP) and of dibutyryl cyclic adenosine monophosphate (db cAMP) on the electrical properties of the isolated toad skin

The addition of db cGMP to the skin (10^{-8}–10^{-6} M) produced a concentration-dependent increase in the bioelectric parameters only at the smaller concentrations: at 10^{-7} M the potential difference increased by $15.0 \pm 2.0\%$ and the short-circuit current increased by $18.0 \pm 1.9\%$ ($P < 0.05$); in most experiments, at 10^{-6} M these values either did not increase significantly or decreased (Fig. 3). When db cAMP in the same concentration range was applied to skins pretreated with db cGMP, additive effects were found only at the smaller concentrations in nearly all the experiments: thus, at 10^{-7} M the potential difference increased by $32.4 \pm 3.8\%$ and the short-circuit current increased by $38.5 \pm 4.2\%$ ($P < 0.05$, $n = 11$).

L-NAME (10^{-6} M) antagonized db cGMP but not db cAMP effects.

3.6. Acute and chronic effects of morphine on the skin responses to several agents

The presence of morphine (10^{-6}–10^{-5} M) applied 15 min previously to the skin antagonized the responses to L-Arg and significantly reduced the responses to carbachol and to acetylchloline. Thus, morphine (10^{-5} M) decreased an L-Arg-induced $24.5 \pm 3.7\%$ ($P < 0.05$) increase in short-circuit current to $4.1 \pm 0.8\%$ and a carbachol-induced $28.4 \pm 2.9\%$ ($P < 0.05$) increase in short-circuit current to $15.0 \pm 2.0\%$ ($n = 6$). The responses to noradrenaline remained unchanged (data not shown). In contrast, when acetylcholine, carbachol or noradrenaline were tested in skins previously incubated 3 h with 10^{-5} M morphine and then carefully washed (see Methods), the responses to cholinergic agents and to noradrenaline were significantly increased (Table 4): that is, a rebound response was observed, suggesting an adaptive phenomenon.

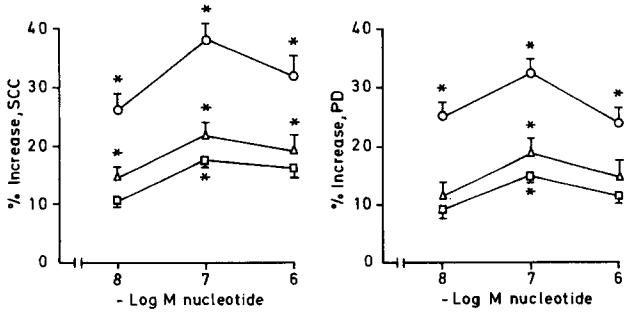

Fig. 3. Additive effects of cGMP and cAMP on the bioelectric parameters of the isolated toad skin. Results are expressed as percentage increase in basal values. Each point represents means ± S.E.M., $n = 11$. SCC, short-circuit current; PD, potential difference; squares, cGMP; triangles, cAMP; circles cGMP plus cAMP. * $P < 0.05$ (analysis of variance and Student-Newman-Keuls test) with respect to basal values.

Table 4
The effect of previous incubation with 10^{-5} M morphine on the toad skin responses to several drugs

Drug	Percentage increase in response			
	Before morphine		After morphine	
	PD (mV)	SCC ($\mu A/cm^2$)	PD (mV)	SCC ($\mu A/cm^2$)
Acetylcholine 10^{-6} M	14.6 ± 3.0 (32.5 ± 3.2)	15.9 ± 3.2 (41.0 ± 3.8)	26.4 ± 4.2 [a]	32.6 ± 4.2 [a]
Carbachol 10^{-6} M	16.4 ± 2.6 (31.7 ± 2.0)	16.8 ± 3.5 (47.7 ± 3.2)	33.6 ± 5.5 [a]	43.0 ± 3.6 [a]
Noradrenaline 10^{-7} M	14.1 ± 2.3 (23.0 ± 3.2)	17.5 ± 2.9 (33.8 ± 3.4)	38.8 ± 4.2 [a]	46.1 ± 4.1 [a]

Values are means ± S.E.M., $n = 8$. PD, potential difference; SCC, short-circuit current. Significantly different from the increase before morphine incubation: [a] $P < 0.01$ (Student's paired t-test). Basal values are indicated in parentheses.

However, the response to L-Arg (3.0×10^{-7} M, $n = 9$) was almost abolished: a $14.3 \pm 2.0\%$ rise in the potential difference and a 16.3 ± 1.7 rise in the short-circuit current ($P < 0.05$) in the naive skin half was reduced to a $1.2 \pm 0.6\%$ and a $2.1 \pm 0.9\%$ rise respectively in the morphine-treated skin half. Control responses to the three agents after 3 h incubation with solvent only were different from values found after incubation with morphine: the response to acetylcholine, carbachol and noradrenaline were similar to the responses before morphine incubation.

The responses to cAMP or cGMP were not affected by the presence of morphine or by previous incubation with the opiate.

4. Discussion

These results show that L-Arg applied to the serosal surface of *Pleurodema thaul* increases potential difference and short-circuit current skin in 69% (53 out of 76) of the experiments carried out. Several studies ascribe the responses to L-Arg in a number of tissues to the synthesis of NO (for review, see Moncada et al., 1991; Goy, 1991).

Previous research in amphibian skin (Turnheim, 1991; Ussing, 1994) has shown that the increase of the electrical parameters of the toad skin involves an enhancement of active Na^+ transport from mucosa to serosa, and also, in *Pleurodema thaul* skin, a possible increase of K^+ transport across the basolateral membrane. Pharmacological evidence that the stimulatory response to L-Arg could be due to increased Na^+ transport is provided by amiloride block;

and support of the existence of K^+ channels in *Pleurodema thaul* skin has been found in previous work where $BaCl_2$ and CsCl decreased the bioelectric parameters of the skin (González et al., 1989; Norris et al., 1994). The similarity of the effects of L-Arg, acetylcholine and carbachol suggest a common mechanism of action in this tissue in accordance with the mechanisms found in mammalian cells (Murad et al., 1978; Kukovetz et al., 1979) where acetylcholine is one of the messengers that triggers NO synthesis. NO activates guanylate cyclase, therefore stimulating the synthesis of cGMP, which is involved in the modulation of several molecular targets (Goy, 1991). The synergism induced by L-Arg on acetylcholine and carbachol effects also suggests that NO pathways may be involved in the skin responses. However, Sahib et al. (1978) working with *Bufo marinus*, found that elevation of cGMP content of the urinary bladder was associated with a decrease in short-circuit current but they did not establish a direct causal relationship; Cuthbert and Wilson (1981) established that acetylcholine in *Rana temporaria* increased net Na^+ flux across isolated toad skin and increased cAMP, but not cGMP, content of the epithelium. Moreover, they did not detect any changes in short-circuit current after addition of cGMP or 8 bromide cGMP, which may reflect species difference. In the present work, the finding that cGMP induced stimulatory responses in *Pleurodema thaul* skin is in favour of the hypothesis that NO is acting through stimulation of this nucleotide.

The L-Arg blocking effects of L-NAME (Rees et al., 1990) and methylene blue (Martin et al., 1985) are accepted as indirect evidence in favour of involvement of NO as second messenger. That both agents antagonized the skin responses to L-Arg and to carbachol, also supports the possibility that NO acts as a messenger in transporting epithelium.

There is no doubt that NO can act by stimulating guanylate cyclase and therefore increasing cGMP levels. Our findings, taken together with the additive effects of cGMP and cAMP could lead to the following hypothesis: K^+ channels are opened in the basolateral membrane by cGMP to stimulate ion transport in this tissue, since 2/3 of the short-circuit current of the toad skin may be carried by K^+ ions (Nielsen, 1984), and cGMP-stimulated cAMP might also activate the insertion of new apical Na^+ channels. The second mechanism is in agreement with the work of Rytved et al. (1995) in frog skin epithelium: in their work, an increased release of prostaglandin E raised cellular cAMP level, leading to an increment in apical Na^+ permeability.

The involvement of NO and cGMP in opiate responses was suggested by Tseng et al. (1992) who demonstrated that the antinociceptive effect of ß-endorphin is increased by pretreatment of mice with L-Arg. In our study the increase in acetylcholine and carbachol responses observed after incubation of the skin with morphine suggests the induction of an adaptive response involving cGMP which may account for the increased responses to these muscarinic agents. These results agree with the findings of Gwynn and Costa (1982) and those of Muraki et al. (1984) who demonstrated an opiate-induced increase of cGMP formation in neuroblastoma cells and in peripheral tissues of C57BL mouse, respectively. On the other hand, the effects of opiates in the adenylate cyclase system are well documented. A rebound increase in cAMP synthesis has been described in a number of tissues (Sharma et al., 1977; Collier, 1980; for review see Cox, 1993), therefore, the observed increment in the responses to noradrenaline after morphine incubation suggests the existence of a morphine-induced adaptive process in the synthesis of both nucleotides in the isolated skin of *Pleurodema thaul*, which could be responsible for the induced supersensitivity.

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