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European Journal of Pharmacology 294 (1995) 451–457

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Sodium fluoride attenuates the negative inotropic effects of muscarinic M_2 and adenosine receptor agonists

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Received 16 March 1995; revised 30 August 1995; accepted 5 September 1995

Abstract

Sodium fluoride increased the force of contraction in isolated guinea-pig papillary muscles concentration dependently, starting at 3 mmol/l. Sodium fluoride inhibited phosphorylase phosphatase activity in homogenates from guinea pig hearts, starting at 1 mmol/l. The positive inotropic effect of 3 mmol/l sodium fluoride was not accompanied by an increase in cAMP content in guinea-pig papillary muscles. In papillary muscles, carbachol or (−)-N^6-phenylisopropyladenosine reduced the positive inotropic effect of isoprenaline (10 nmol/l) or the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (60 μmol/l). These negative inotropic effects of carbachol and (−)-N^6-phenylisopropyladenosine were attenuated by additional sodium fluoride (3 mmol/l). It is concluded that sodium fluoride can impair the signal transduction of muscarinic M_2 (carbachol) and adenosine receptor ((−)-N^6-phenylisopropyladenosine) agonists. This effect of sodium fluoride could support the hypothesis that the cardiac effects of muscarinic M_2 and adenosine receptor agonists involve, at least in part, the activation of phosphatases.

Keywords: Sodium fluoride; Carbachol; (−)-N^6-Phenylisopropyl-adenosine; Phosphatase; Inotropy

1. Introduction

The positive inotropic and lusitropic effects of sympathetic stimuli are attenuated by parasympathetic stimuli (Löffelholz and Pappano, 1985; Hartzell, 1988) and similarily by adenosine (Olsson and Pearson, 1990).

The negative inotropic effects of acetylcholine (via muscarinic M_2 receptors) and adenosine (via adenosine A_1 receptors, for brevity: adenosine receptor) in the mammalian ventricle are enhanced in the presence of cAMP-increasing agents (Dobson, 1978, 1983; Böhm et al., 1984; Endoh, 1987). Acetylcholine and adenosine receptor agonists can inhibit adenylyl cyclase activity in broken cardiac cell preparations (Watanabe and Besch, 1975a; Baumann et al., 1981; Heller et al., 1989). It is, however, controversial whether muscarinic M_2 and adenosine receptor agonists actually reduce the isoprenaline-stimulated cardiac cAMP content in intact preparations. In spontaneously beating guinea-pig ventricles, rat ventricles, rat cardiomyocytes, rabbit and dog ventricular preparations, acetylcholine and/or adenosine derivatives reduce the isoprenaline-stimulated cAMP content (Baumann et al., 1981; Dobson, 1978, 1983; Endoh, 1987; Endoh et al., 1991; George et al., 1991). Others have failed to detect the reduction of isoprenaline- or 3-isobutyl-1-methylxanthine (IBMX)-stimulated cAMP content by acetylcholine or (−)-N^6-phenylisopropyladenosine (R-PIA) in guinea-pig and chicken cardiac ventricular preparations (Biegon et al., 1980; Watanabe and Besch, 1975b; Böhm et al., 1984, 1988; Lindemann and Watanabe, 1985; Schmied and Korth, 1990; Neumann et al., 1994a, 1995).

There is convincing evidence that acetylcholine and adenosine can affect protein phosphorylation in perfused hearts and other cardiac preparations. For instance, England (1976) has shown that acetylcholine can reduce isoprenaline-stimulated phosphorylation of the inhibitory subunit of troponin in perfused rat hearts. Likewise, acetylcholine and R-PIA reduced isoprenaline-stimulated phospholamban phosphorylation in perfused guinea-pig hearts (Lindemann and Watanabe, 1985; Neumann et al., 1995). Others have reported that adenosine receptor and muscarinic M_2 receptor agonists reduce phospholamban phosphorylation in

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SSDI 0014-2999(95)00569-2

chicken ventricles (Iwasa and Hosey, 1983; Hosey et al., 1984). Furthermore, adenosine receptor and muscarinic M_2 receptor agonists reduce isoprenaline-stimulated phosphorylation in isolated cardiomyocytes (George et al., 1991; Gupta et al., 1994). The negative inotropic effect of adenosine and muscarinic M_2 receptor activation is accompanied by an activation of protein phosphatase activities in guinea-pig ventricular preparations (Ahmad et al., 1989; Gupta et al., 1993). Hence, if this activation of phosphatases is functionally important, we hypothesized-that an inhibitor of protein phosphatases (for brief, phosphatases) might attenuate the inotropic effects of adenosine receptor and muscarinic M_2 receptor activation. Sodium fluoride is one of the classical phosphatase inhibitors (cf. Shenolikar and Nairn, 1991).

Therefore, in the present study the influence of sodium fluoride on the negative inotropic effects of muscarinic M_2 (by carbachol) and adenosine receptor stimulation (by R-PIA) in the presence of cAMP-increasing agents in the mammalian ventricle was investigated.

2. Materials and methods

2.1. Determination of inotropic response

Contraction experiments were performed as described previously (Böhm et al., 1984). In brief, papillary muscles were isolated from right ventricles of reserpinized (5 mg/kg, 16 h before the animals were killed) guinea pigs. The bathing solution contained (in mmol/l) NaCl 119.8, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 22.6, Na_2EDTA 0.05, ascorbic acid 0.28, glucose 5.05, continuously gassed with 95% O_2 and 5% CO_2 and was maintained at 35°C and pH 7.4. Isometric force of contraction was measured after each muscle was preloaded to an optimal length. Preparations were electrically stimulated at 1 Hz with rectangular pulses of 5 ms duration (Grass stimulator SD9; Grass, Quincy, MA, USA), the voltage was about 10–20% greater than threshold. Preparations were allowed to equilibrate for 30 min. Thereafter the drugs were added. The order and times of addition are exemplified in Fig. 3. As indicated in the appropriate legends, compounds were added alone or cumulatively.

2.2. Preparation of homogenates and membranes

Homogenates and membranes were prepared as described previously (Neumann et al., 1993). In brief, guinea-pig ventricles were freeze-clamped. Powdered tissue was homogenized in 10 ml medium containing (in mmol/l) 4.0 EDTA and 0.1% β-mercaptoethanol (v/v). The tissue was thawed and homogenized 3 times for 30 s each with a Polytron PT-10 (Kinematica, Lucerne, Switzerland). The sample was sedimented for 20 min at $14000 \times g$. The resultant supernatant is termed homogenate. In further studies, the supernatant was sedimented at $45000 \times g$ for 30 min, and the resultant pellet was resuspended in 10 ml of homogenization medium containing 0.6 M NaCl. This material was sedimented at $45000 \times g$ for 30 min. The final pellet containing the membrane vesicles was resuspended in 200 μl of 50 mmol/l tris(hydroxymethyl) aminomethane (Tris) · HCl (pH 7.0), 0.1 mmol/l EDTA, and 0.1% β-mercaptoethanol.

2.3. Protein phosphatase assay

Assays for protein phosphatase activity were performed exactly as described (Neumann et al., 1993). Phosphatase activity was measured at 30°C using [^{32}p] phosphorylase a as substrate. The 50 μl incubation mixture contained (in mmol/l) 20.0 Tris · HCl (pH 7.0), 5.0 caffeine, 0.1 EDTA and 0.1% β-mercaptoethanol (v/v). The reaction was started by addition of homogenate or membrane vesicles. The reaction was terminated after 10 min by addition of trichloroacetic acid. Samples were centrifuged and radioactivity in the supernatants was determined.

2.4. Determination of cAMP content

Determination of cAMP content in papillary muscles was performed as described (Böhm et al., 1988). Briefly, papillary muscles were prepared and stimulated as described above. Thereafter, contracting muscles were incubated with 3 mmol/l sodium fluoride for 30 min and then freeze-clamped and the cAMP content was determined by means of a radioimmunoassay (Böhm et al., 1984). Control muscles were treated similarily.

2.5. Chemicals

The compounds used were (−)-N^6-phenylisopropyladenosine (R-PIA, Boehringer Mannheim, Germany), sodium fluoride (Merck, Darmstadt, Germany), (±) isoprenaline hydrochloride (Boehringer Ingelheim, Ingelheim, Germany) 3-isobutyl-1-methylxanthine (IBMX), carbamylcholine chloride (both from Sigma, Munich, Germany), ^{125}I-labeled sodium iodide and [γ-^{32}P]ATP (both from Amersham Buchler, Braunschweig, Germany). [^{32}P]Phosphorylase *a* was prepared from phosphorylase *b* (from Sigma, Munich, Germany) as reported (Neumann et al., 1993). All other chemicals were of analytical grade or the best commercial grade available. Deionized and twice-distilled water was used throughout.

2.6. Statistics

Data are given as means ± S.E.M. The significance of differences was tested using Student's *t*-test for paired and unpaired observations as appropriate. $P < 0.05$ was regarded as significant.

3. Results

Fig. 1 shows that sodium fluoride added cumulatively exerted a positive inotropic effect in guinea-pig papillary muscles in a biphasic fashion. In contrast, the same concentrations of sodium chloride failed to affect contractile activity (data not shown). Furthermore, sodium fluoride inhibited concentration dependently, starting at 1 mmol/l, the activity of phosphatases in homogenates from guinea-pig ventricles with an IC_{50} value of about 2.5 mmol/l ($n = 3$). In addition, we chose to measure the phosphatase activity in membrane vesicles (Fig. 2) because they are enriched in functionally relevant phosphoproteins of sarcolemmal and sarcoplasmic origin (Lindemann and Watanabe, 1985; Neumann et al., 1995). Hence, inhibition of phosphatases in membrane vesicles might be functionally more relevant than inhibition in homogenates. Because 3 mmol/l sodium fluoride was the lowest concentration that exerted a positive inotropic effect (Fig. 1) and was able to inhibit, in part, cardiac phosphatase activities (Fig. 2), this concentration was used in the remainder of this study.

Next, we asked whether the positive inotropic effect of sodium fluoride (3 mmol/l) could be due to an increase in cAMP content. Sodium fluoride applied for

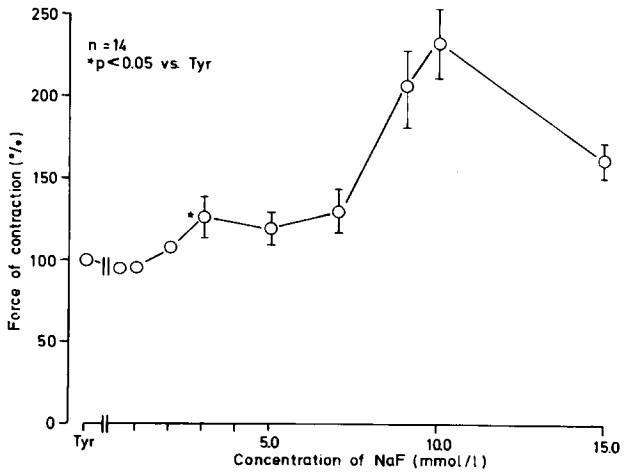

Fig. 1. Effects of cumulatively applied sodium fluoride on force of contraction in guinea-pig papillary muscles. Pre-drug values for force of contraction amounted to 0.69 ± 0.11 mN (milli Newton). * The first significant difference vs. pre-drug value (Tyr). n = number of experiments. Ordinate: force of contraction in percentage of pre-drug value. Abscissa: concentration of sodium fluoride (NaF).

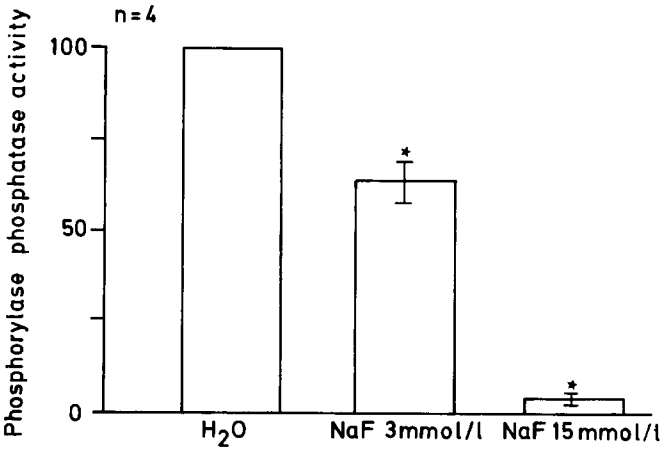

Fig. 2. Effect of sodium fluoride on phosphorylase phosphatase activity in membrane vesicles from guinea-pig ventricles. Membrane vesicles were prepared and phosphorylase phosphatase activity was measured as described in Materials and methods. Ordinate: phosphorylase phosphatase activity as percentage of control. Abscissa: concentrations of sodium fluoride (NaF). * Significant differences versus control (= H_2O).

30 min (a time used in all subsequent experiments) did not increase the cAMP content in papillary muscles (sodium fluoride: 1.11 ± 0.12 pmol/mg wet weight, $n = 8$, versus control conditions: 1.16 ± 0.13 pmol/mg wet weight, $n = 7$). In addition, sodium fluoride did not shorten the duration of contraction (data not shown), unlike isoprenaline.

Thereafter, we studied whether sodium fluoride could affect the negative inotropic effect of carbachol or R-PIA. Carbachol or R-PIA alone did not decrease the force of contraction. Even after pretreatment of papillary muscles for 30 min with 3 mmol/1 sodium fluoride, 1 μmol/l carbachol or 1 μmol/1 R-PIA did not decrease the force of contraction (data not shown). In contrast, it is well known that after treatment of ventricular preparations with cAMP increasing agents like isoprenaline (Figs. 3A, 4 and 5) or IBMX (Figs. 6 and 7), both carbachol and R-PIA reduce the force of contraction. Actually, in order to facilitate comparison with earlier work, the same concentrations of isoprenaline (10 nmol/l) and IBMX (60 μmol/l) were used as before (e.g. Böhm et al., 1988; Gupta et al., 1993).

When papillary muscles were pretreated with 3 mmol/l sodium fluoride (but not with sodium chloride, data not shown), the negative inotropic effect of 1 μmol/l carbachol in the presence of isoprenaline was nearly abolished. This is exemplified in Fig. 3B. One may expect that the positive inotropic effect of isoprenaline would be enhanced in the presence of sodium fluoride. Surprisingly, this was not the case (cf. Figs. 3, 4 and 5). At present we can only speculate that e.g. sodium fluoride might inhibit phosphatase activities and thus potentiate the effect of protein kinases that

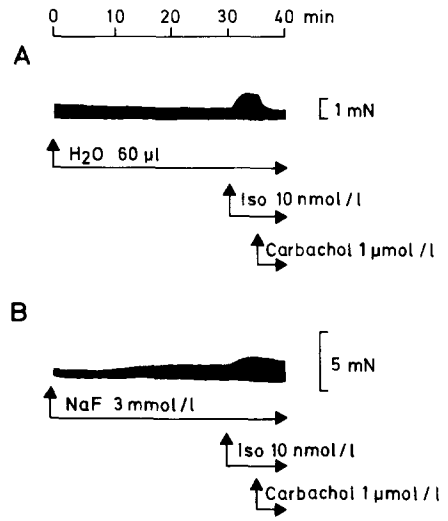

Fig. 3. Original recording depicts the influence of sodium fluoride on the negative inotropic effect of carbachol. Isolated electrically driven guinea-pig papillary muscles were treated first with sodium fluoride (NaF, B) or water (A) for 30 min. Thereafter, isoprenaline (Iso) and subsequently carbachol were applied at the times indicated (compare time bar).

reduce β-adrenoceptor function. Thus, indirectly, sodium fluoride might potentiate the action of the β-adrenoceptor kinase or the cAMP-dependent kinase – and hence inhibit (at least not potentiate) the action of β-adrenoceptor agonists (for review, see Bylund et al., 1994). Data from several experiments are summarized in Fig. 4. The concentration-dependent effect of the muscarinic M_2 receptor agonist carbachol in the

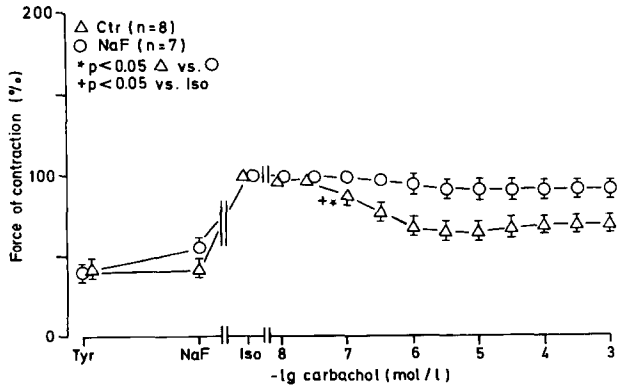

Fig. 4. Effects of carbachol on isoprenaline (Iso)-stimulated force of contraction in the absence (Ctr) or presence of sodium fluoride (NaF). The times of incubation and concentrations of compounds were the same as in Fig. 3. The effects of sodium fluoride were quantified 30 min after addition. The effects of various concentrations of carbachol (cumulatively applied) were measured 5 min after addition of each concentration. Pre-drug (Tyr) values for force of contraction amounted to 1.23 ± 0.17 mN. * The first significant difference vs. sodium fluoride-treated muscles. + The first significant difference versus the effect of isoprenaline (Iso). n = number of experiments. Ordinate: force of contraction in isolated guinea-pig papillary muscles in percentage of the effect of isoprenaline (Iso, 5 min).

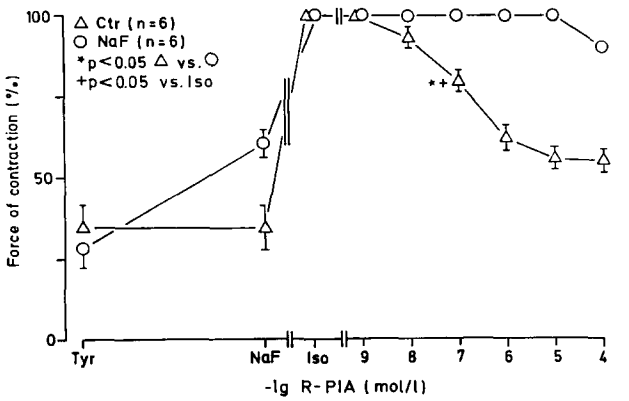

Fig. 5. Effects of R-PIA on isoprenaline (Iso)-stimulated force of contraction in the absence (Ctr) or presence of sodium fluoride (NaF). The times of incubation and concentrations of compounds were the same as in Fig. 7. The effects of sodium fluoride were quantified 30 min after its addition. The effects of various concentrations of R-PIA (cumulatively applied) were measured 5 min after addition of each concentration. Pre-drug values (Tyr) for force of contraction amounted to 1.33 ± 0.28 mN. * The first significant difference vs. sodium fluoride-treated muscles. + The first significant difference versus the effect of isoprenaline (Iso). n = number of experiments. Ordinate: force of contraction in isolated guinea-pig papillary muscles in percentage of the effect of isoprenaline (Iso, 5 min).

presence of isoprenaline was greatly attenuated by sodium fluoride. Subsequently, the effect of sodium fluoride on the negative inotropic effect of the adenosine receptor agonist R-PIA was studied. The effect of sodium fluoride on the concentration-dependent effect of R-PIA in the presence of isoprenaline is summarized in Fig. 5. Apparently, the effect of R-PIA can be

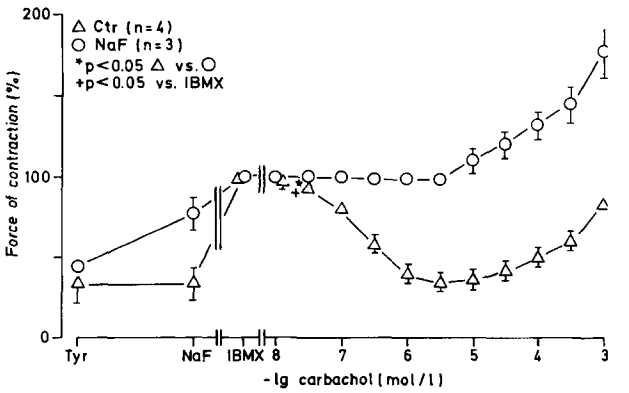

Fig. 6. Effects of carbachol on 3-isobutyl-1-methylxanthine (IBMX)-stimulated force of contraction in the absence (Ctr) or presence of sodium fluoride. The basic protocol was the same as in Fig. 3. The effects of sodium fluoride were quantified 30 min after its addition. The effects of various concentrations of carbachol (cumulatively applied) were measured 5 min after addition of each concentration. Pre-drug values for force of contraction amounted to 1.07 ± 0.18 mN. * The first significant difference vs. sodium fluoride treated muscles. + The first significant difference versus the effect of IBMX. n = number of experiments. Ordinate: force of contraction in isolated guinea-pig papillary muscles in percentage of the effect of IBMX (15 min, 60 μmol/l).

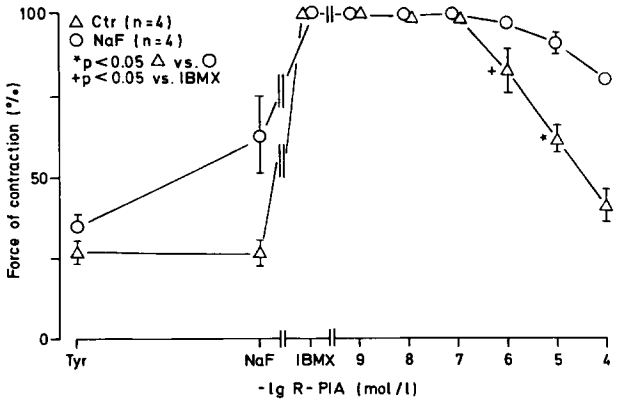

Fig. 7. Effects of R-PIA on 3-isobutyl-1-methylxanthine (IBMX)-stimulated force of contraction in the absence (Ctr) or presence of sodium fluoride. The effects of sodium fluoride were quantified 30 min after its addition. The effects of various concentrations of R-PIA (cumulatively applied) were measured 5 min after addition of each concentration. Pre-drug values for force of contraction amounted to 1.45 ± 0.12 mN. * The first significant difference vs. sodium fluoride-treated muscles. + The first significant difference versus the effect of IBMX. n = number of experiments. Ordinate: force of contraction in isolated guinea-pig papillary muscles in percentage of the effect of IMBX (15 min, 60 μmol/l).

significantly reduced by pretreatment of papillary muscles with sodium fluoride.

Isoprenaline increases the cAMP content directly via a receptor-mediated mechanism. It could be asked whether or not sodium fluoride can affect effects elicited in the presence of receptor-independent elevations of cAMP content. One useful tool for increasing cAMP content independently of β-adrenoceptor activation is the phosphodiesterase inhibitor IBMX (Böhm et al., 1988). It turned out that sodium fluoride was able to attenuate the negative inotropic effects of carbachol (Fig. 6) and R-PIA (Fig. 7) under these conditions. Very high (nonphysiological) concentrations of carbachol induced a significant positive inotropic effect (Fig. 6). This well-known positive inotropic effect of carbachol at high concentrations (Löffelholz and Pappano, 1985; Pappano, 1991) has been attributed to an activation of phospholipase C (Kohl et al., 1990). However, it is not known whether carbachol stimulates phospholipase C to a greater extent in the presence or in the absence of IBMX, as this hypothesis would postulate.

4. Discussion

The main new finding of this study was the observation that sodium fluoride at a concentration that inhibits protein phosphatase activity (in part) in a cardiac broken cell preparation can attenuate the negative inotropic effects of adenosine receptor agonists and muscarinic M_2 receptor agonists, effects which are only observed in the presence of cAMP-increasing agents.

It is still controversial whether the negative inotropic effects of adenosine receptor and muscarinic M_2 receptor agonists are mediated exclusively via an inhibition of adenylyl cyclase activity and a reduction of cAMP content. It is quite possible that different experimental conditions and species differences can account for the apparent discrepancies in the literature (see Introduction). It is generally agreed that adenosine receptor and muscarinic M_2 receptor agonists reduce the phosphorylation state of regulatory proteins like phospholamban, phospholemman, the inhibitory subunit of troponin and C-protein (England, 1976; George et al., 1991; Neumann et al., 1994a; Gupta et al., 1994; Neumann et al., 1995).

This reduced phosphorylation state could be due to an inhibition of protein kinase A activity. However, we failed to detect an inhibition of isoprenaline-stimulated protein kinase A activity by adenosine receptor or muscarinic M_2 receptor agonists (Gupta et al., 1993). In contrast, the dephosphorylation could be due to an increased phosphatase activity. Indeed, direct (Ahmad et al., 1989) and indirect evidence (Gupta et al., 1993, 1994) indicates that adenosine receptor and muscarinic M_2 receptor agonists can increase phosphatase activities in preparations from guinea-pig cardiac ventricles.

Thus, one can ask whether inhibition of phosphatases will attenuate the negative inotropic effects of adenosine receptor and muscarinic M_2 receptor agonists.

Cardiac phosphatases can be divided into type 1, 2A, 2B and 2C. Several inhibitors for type 1 and 2A cardiac phosphatase are available, namely okadaic acid (Neumann et al., 1993) and calyculin A (Neumann et al., 1994b). Sodium fluoride inhibits type 1, 2A and 2B but not 2C phosphatase activities (for review, see Shenolikar and Nairn, 1991). Hence, the broad inhibitory spectrum makes it tempting to try sodium fluoride.

It has been shown before that sodium fluoride is a positive inotropic agent (Loewi, 1955; Vogel et al., 1977). Sodium fluoride stimulates the slow Ca^{2+} currents in embryonic chick heart cells (Vogel et al., 1977). These effects could be due, for instance, to inhibition of phosphatase activity or activation of adenylyl cyclase activity. Sodium fluoride will activate adenylyl cyclase activity in broken cell preparations (Rall and Sutherland, 1958). This effect has been attributed to the activation of the stimulatory GTP binding protein, Gs, which activates adenylyl cyclase activity (Howlett et al., 1979; Katada et al., 1984). However, if this were the case in papillary muscles, one would expect an increase in cAMP content – which we failed to observe (Fig. 3). Likewise, others reported that sodium fluoride did not increase the cAMP content in the avian heart and in bovine smooth muscle preparations (Vogel et al., 1977; Hall et al., 1990). In addition,

unlike cAMP increasing agents, sodium fluoride did not shorten the duration of single muscle contractions and the inotropic effects of sodium fluoride were not decreased by carbachol (see Results). Still, the increase might affect a compartment which we failed to measure (Buxton and Brunton, 1983) or might be too small to be detectable by the methods employed.

There is agreement that pertussis toxin pretreatment abolishes the inotropic, electrophysiological, biochemical (Hazeki and Ui, 1981; Pfaffinger et al., 1985; Endoh et al., 1985; Böhm et al., 1986; Kurachi et al., 1986) and phosphorylation effects of acetylcholine and adenosine receptor agonists in the presence of isoprenaline in ventricular preparations (Neumann et al., 1994a). Sodium fluoride can interact with pertussis toxin-sensitive GTP-binding proteins (Katada et al., 1984). Hence, sodium fluoride might inhibit the function of pertussis toxin-sensitive GTP binding proteins which mediate the physiological functions of acetylcholine and adenosine in the heart. However, since the cAMP-independent effect of these agonists is small in the mammalian ventricular myocardium, and in addition because the change in cAMP levels does not correlate to the functional regulation in the guinea-pig heart, this mechanism may be less important in this animal species.

In addition, sodium fluoride has several other cellular effects. For instance, sodium fluoride activates phospholipase C in hepatocytes (Blackmore et al., 1985). It can inhibit and stimulate the Ca^{2+} pump of the sarcoplasmic reticulum (Narayanan et al., 1991). Sodium fluoride inhibits the sarcolemmal Na^+,K^+-ATPase (for instance: Murphy and Hoover, 1992). In addition, other as yet unknown proteins might be affected by sodium fluoride treatment.

Thus, sodium fluoride could interfere with a host of pathways. However, it is still possible to speculate that sodium fluoride inhibits phosphatases, for instance in the sarcolemma, that dephosphorylate cardiac proteins after stimulation of receptors. Indeed, we have shown that sodium fluoride attenuates the effects of muscarinic M_2 receptor activation in the presence of isoprenaline on L-type Ca^{2+} channels in guinea-pig ventricular myocytes (Herzig et al., 1995). It is noteworthy that sodium fluoride is about 10 times more potent in antagonizing the effects of isoprenaline than antagonizing those of IBMX. One can speculate that localization may play a role. Isoprenaline acts in the membrane to increase cAMP production whereas IBMX most probably acts more within the cell cytosol by inhibiting phosphodiesterase activity. Thus, sodium fluoride might simply have a higher local concentration in the membrane than in the cytosol and this might explain the observed difference in potency. However, interference with additional specific target proteins for sodium fluoride (besides phosphatases) may contribute to this effect. Nevertheless, it is likely that the effects of sodium fluoride reported here are mainly due to an inhibition of phosphatase activities.

Whatever the relevant mechanism of action of sodium fluoride might be, our observations could be of clinical relevance. Sodium fluoride intoxication can lead to ventricular irritability (McIvor et al., 1987). This is usually explained by hyperkalemia due to inactivation of Na^+,K^+-ATPase in erythrocytes. However, the situation might be aggravated by the inability of vagal stimuli to antagonize the effects of sympathetic stimuli.

In summary, sodium fluoride, a protein phosphatase inhibitor, attenuates the negative inotropic effects of adenosine receptor and muscarinic M_2 receptor agonists in the presence of cAMP-increasing agents in the mammalian heart. These results do not contradict the hypothesis that some of the effects of adenosine receptor and muscarinic M_2 receptor agonists are mediated via activation of protein phosphatase(s).

Acknowledgement

This study was supported by the DFG.

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