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SENSITIVITY OF THE RAT DIAPHRAGM ADRENERGIC RECEPTORS

THE RESPONSE OF THE PHOSPHORYLASE SYSTEM TO STIMULATION BY VARIOUS ADRENERGIC AGONISTS IN THE PRESENCE OF IODOACETATE OR PYRUVATE

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Abstract—The effects of iodoacetate and pyruvate on the response of the rat diaphragm phosphorylase system to beta-1 and beta-2 adrenergic stimulation have been investigated. It was observed that terbutaline was more effective than noradrenaline in stimulating the conversion of phosphorylase *b* to *a*. The stimulation by both of these agents was antagonized much more effectively by butoxamine than by practolol. No additive effects of butoxamine and practolol against noradrenaline or terbutaline were observed. Iodoacetate potentiated adrenergic stimulation of phosphorylase conversion, the order of potency being terbutaline > adrenaline > noradrenaline. Pyruvate, which inhibits the effect of beta adrenergic agents on the phosphorylase system, was more effective against the action of noradrenaline than in opposing terbutaline stimulation of phosphorylase *b* to *a* conversion. The results indicate that adrenergic agonists promote formation of phorphorylase *a* in diaphragm muscle by beta-2 receptor activation. It is, however, suggested that beta-1 and beta-2 agonists have different modes of interaction with this receptor.

We have previously presented data which indicated that the adrenaline stimulated conversion of phosphorylase *b* to *a* in skeletal muscle is subject to metabolic regulation [1, 2]. Both iodoacetate and fluoroacetate were found to potentiate the effect of adrenaline on the skeletal muscle phosphorylase system. This effect of iodoacetate was completely abolished by the presence of pyruvate which also partially prevented the stimulatory action of fluoroacetate. Pyruvate alone inhibited the response of the phosphorylase system to adrenaline. Phosphorylase conversion induced by tetanic electrical stimulation or dibutyryl cyclic AMP was modified in a similar manner. The levels of cyclic AMP achieved following adrenergic stimulation were decreased by the presence of iodoacetate and were not influenced by pyruvate. These data supported the view that the conversion of phosphorylase is under the control of at least two systems, the well established cyclic AMP protein kinase system and another which may involve fluctuations in the concentration of free intracellular Ca^{2+} [3–6]. We have suggested that the latter system can be influenced by metabolic inhibitors and pyruvate. Indirect support for this was found in studies of glycogen synthetase. The activation of this enzyme is not dependent upon Ca^{2+} and was unaffected by the presence of iodoacetate or pyruvate.

Our studies on metabolic control of adrenergic stimulated phosphorylase conversion have been extended by investigating the effects of iodoacetate and pyruvate on stimulation by various adrenergic agonists. Previous studies in which stimulation of contractility was used as a measure of agonist action indicated that the adrenergic receptors of the rat diaphragm belong to the beta group and are of the beta-2 subtype [7]. It has, however, been suggested that adrenergic receptors may be interconvertible and that the transition might be dependent upon the metabolic state of the tissue [12–14]. We have, therefore, investigated the character of the diaphragm adrenergic receptors under conditions of differing metabolic patterns, using phosphorylase conversion as an indication of receptor–agonist interaction. The differing metabolic patterns were produced by incubation with glucose as the only substrate in the absence of insulin, with glucose plus excess pyruvate and with glucose in the presence of iodoacetate. In each situation the effects of noradrenaline (alpha and beta-1 agonists), terbutaline (beta-2 agonist) and adrenaline (alpha, beta-1, beta-2) upon diaphragm phosphorylase conversion were investigated. The results confirm earlier studies which demonstrated that adrenergic receptors of rat diaphragm are of the beta-2 type. However, the data presented in this communication suggest that the signal arising from adrenergic receptor interaction is dependent upon the structure of the agonist.

MATERIALS AND METHODS

Incubation. Male Wistar-derived rats weighing 120–150 g were used in this study. The animals were stunned and killed by decapitation and the diaphragms were rapidly dissected out with the ribs attached. These preparations were then divided to yield intact hemidiaphragms. The tissues were placed in ice-cold Krebs–Ringer bicarbonate buffer of the following composition: 119 mM NaCl, 4·7 mM KCl, 1·2 mM $MgSO_4$, 2·6 mM $CaCl_2$, 1·2 mM KH_2PO_4, 25 mM $NaHCO_3$, 5 mM glucose. The solution was

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Table 1. Effects of butoxamine and practolol on terbutaline and noradrenaline stimulated conversion of phosphorylase

Addition to medium	Per cent phosphorylase *a*		
	Basal	Terbutaline 3×10^{-7} M	Noradrenaline 6×10^{-6} M
None	7·8 ± 0·7 (11)*	33·0 ± 1·8 (4)	29·9 ± 2·2 (8)
Butoxamine 5×10^{-7} M	8·6 ± 0·5 (4)	21·6 ± 0·9 (6)†	20·0 ± 2·0 (4)†
Practolol $1·5 \times 10^{-4}$ M	6·8 ± 0·4 (4)	16·6 ± 1·4 (10)†	23·2 ± 2·0 (6)†
Butoxamine 5×10^{-7} M + practolol $1·5 \times 10^{-7}$ M	—	17·5 ± 2·4 (4)†	22·3 ± 0·9 (4)†

* Mean value ± S.E.M. with number of experiments in parentheses.

† $P < 0·05$ compared to phosphorylase levels obtained without additions to the medium.

Intact hemidiaphragms were incubated for 15 min in Krebs–Ringer bicarbonate buffer containing 5 mM glucose. Butoxamine or practolol were present as indicated. At the end of the incubation period terbutaline or noradrenaline was added. The incubation was continued for 5 min in the presence of agonist.

equilibrated with 95% O_2–5% CO_2 at 37°. The tissues were equilibrated for 15 min at 37° in 20 ml Krebs–Ringer bicarbonate buffer. Pyruvate (20 mM), iodoacetate (1 mM), practolol, butoxamine or phentolamine were added to the medium when appropriate. Terbutaline, adrenaline or noradrenaline were added at the end of the preincubation period. The final concentrations of the various pharmacologic agents are indicated in the accompanying tables and figures. After incubation samples (40–80 mg) of the diaphragms were rapidly frozen in liquid nitrogen.

Homogenization of the tissues and determination of phosphorylase activity was carried out as previously described [1].

Statistical significance was evaluated by Student's *t*-test.

Chemicals. Terbutaline sulphate (KWD 2019, Batch 10) was a gift from Draco, Sweden. Adrenaline was obtained from Societé des Usines Chimiques, Rhône-Poulenc, Paris, and *l*-noradrenaline *d*-bitartrate monohydrate (Batch no. 12583) from N. V. Philips-Roxane WEESP, Holland. Practolol (ICI 50, 172 ADM 11800/70) and butoxamine (46982) were the kind gifts of ICI, England, and Dr. J. Shanfeld, University of Pennsylvania, respectively.

Terbutaline, adrenaline, noradrenaline and butoxamine were dissolved in 20 mM HCl. Practolol was dissolved by adding HCl to pH 1·5 and, after neutralization, further dilution was made in 20 mM HCl. Fresh solutions were prepared each day.

RESULTS

Effects of butoxamine and practolol on terbutaline and noradrenaline stimulated phosphorylase conversion. Experiments were performed with selective agonists and antagonists in order to obtain information concerning the nature of the diaphragm adrenoreceptor involved in phosphorylase activation. Preliminary studies indicated that 6×10^{-6} M noradrenaline stimulated phosphorylase conversion to the same degree as 3×10^{-7} M terbutaline (Table 1). The responses to terbutaline and noradrenaline were inhibited to the same extent by 5×10^{-7} M butoxamine. A 300-times higher concentration of practolol had to be used in order to obtain a similar degree of inhibition. No additive effects of butoxamine and practolol were observed. Phentolamine (10^{-5} M) had no influence on the effects of noradrenaline. Basal phosphorylase *a* activity was not influenced by the antagonists and total (*a* + *b*) phosphorylase activity remained unaltered in the presence of the various agents.

Relative potencies of pyruvate and iodoacetate on the responses of the phosphorylase system to noradrenaline, adrenaline and terbutaline. The relative potencies of pyruvate and iodoacetate on the stimulation of phosphorylase conversion by noradrenaline, adrenaline and terbutaline are shown in Fig. 1. It should be pointed out that the control values for phosphorylase *a* production (i.e. in the absence of iodoacetate and pyruvate) were very similar in all three groups as can be seen in the legend. Furthermore, the level of stimulation is submaximal, 6×10^{-5} M noradrenaline gave 51·2 ± 0·9 per cent phosphorylase *a* (n = 6) in the absence of iodoacetate or pyruvate. Increasing the concentration of terbutaline to 3×10^{-6} M gave 51·6 ± 5·0 per cent phosphorylase *a* (n = 6) under these conditions. It can be seen from Fig. 1 that

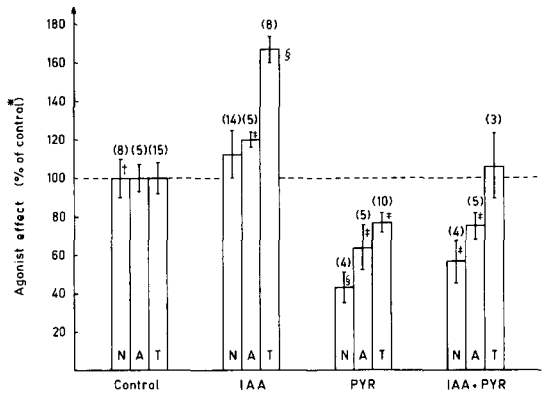

Fig. 1. Relative potencies of pyruvate and iodoacetate on the responses of the phosphorylase system to noradrenaline, adrenaline and terbutaline. Hemidiaphragms were incubated for 15 min in the absence (control) or presence of iodoacetate, 1 mM (IAA), pyruvate, 20 mM (PYR) or both (IAA + PYR). The agonists were then added and the incubations were continued for 5 min. N = noradrenaline (6×10^{-6} M), A = adrenaline (6×10^{-6} M), T = terbutaline (3×10^{-7} M). * In control experiments (i.e. in the absence of iodoacetate or pyruvate) the effects of the agonists on the phosphorylase system (i.e. the difference between % phosphorylase *a* in the presence of agonist and basal % *a*) were 22·1, 21·6 and 22·9 for noradrenaline, adrenaline and terbutaline, respectively. † Mean ± S.E.M. with number of experiments in parentheses. ‡ $P < 0·05$ versus control. § $P < 0·001$ versus control.

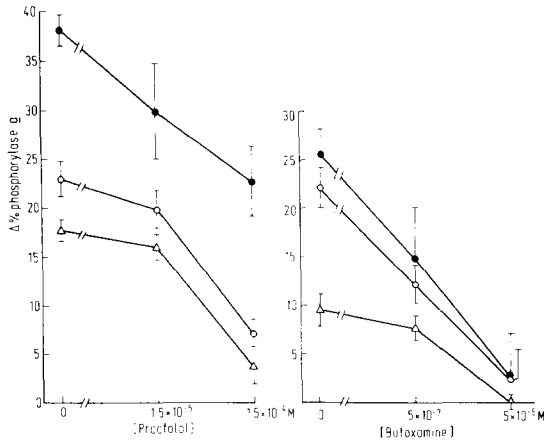

Fig. 2. Effects of iodoacetate and pyruvate on terbutaline and noradrenaline stimulated conversion of phosphorylase *b* to *a* in the presence of beta blockers. Hemidiaphragms were incubated for 15 min in the presence of beta blockers and iodoacetate (1 mM) or pyruvate (20 mM) as indicated. Terbutaline (left) or noradrenaline (right) was added and the incubation was continued for 5 min. Each point represents the mean value of 4–10 experiments. 2 S.E.M. are indicated by the vertical bars. Final concentrations of terbutaline and noradrenaline were 3×10^{-7} M and 6×10^{-6} M, respectively; O——O, control; ●——●, iodoacetate; △——△, pyruvate.

the effectiveness of both iodoacetate and pyruvate varied greatly from one agonist to the other. Iodoacetate did not cause any significant potentiation of noradrenaline stimulation of phosphorylase conversion, but caused a small potentiation of the effect of adrenaline and marked potentiation of terbutaline stimulated conversion of phosphorylase *b* to *a*. An opposite order of potency was found for pyruvate, the effect of which being most pronounced on the response to noradrenaline and least on terbutaline. The series of experiments in which both iodoacetate and pyruvate were present gave values lying between those found in the presence of only one of these substances. It should be pointed out that the relative effectiveness of the adrenergic agonists did not change under the different experimental conditions.

Effects of iodoacetate and pyruvate on terbutaline and noradrenaline stimulated conversion of phosphorylase b *to* a *in the presence of beta blockers.* In order to increase the selectivity of beta receptor stimulation we used the beta-2 agonist terbutaline in the presence of a beta-1 blocker practolol (Fig. 2, left). To obtain a relatively specific beta-1 stimulation we used the beta-1 agonist noradrenaline with the beta-2 blocker butoxamine (Fig. 2, right). It can be seen from Fig. 2 that high concentrations of practolol were required to inhibit the response of the phosphorylase system to terbutaline. The marked increase in phosphorylase conversion observed in the presence of iodoacetate was not prevented by practolol. Similarly, the inhibition by pyruvate of the response to terbutaline appeared to be little affected by practolol. Butoxamine at low concentrations inhibited the response of the phosphorylase system to noradrenaline in the absence and presence of iodoacetate. The marked inhibition produced by pyruvate was not additive to the effect of butoxamine (Fig. 2, right).

DISCUSSION

The classification of adrenergic receptors as alpha and beta was proposed in 1948 by Ahlquist [8] and is based on the differing potencies of a series of catecholamines. The adrenoreceptors classified as beta are not homogenous and it has been suggested that they can be further divided into the subgroups beta-1 and beta-2 [9–11]. Lands *et al.* [7] have studied the nature of the beta receptor of isolated diaphragm strips using augmentation of contraction as an indication of receptor activation. These workers concluded that the adrenergic receptor of this preparation is of the beta-2 type. In a recent review article, Nickerson [12] has discussed evidence which indicates that alpha and beta receptors are interconvertible and that the transition might be dependent upon the metabolic state of the tissue in question. A similar hypothesis concerning the specificity of the subdivisions of the beta receptor has been set forward by Wagner *et al.* [13] and by Schümann *et al.* [14].

The order of potency of beta-1 and beta-2 agonists and antagonists found in this study is such that one can define the receptor as being of the beta-2 type in complete agreement with Lands *et al.* [7]. However, it appears that this definition is not sufficiently precise to characterize this receptor completely. A striking difference between the response to terbutaline and noradrenaline was observed when tissues were incubated with iodoacetate or pyruvate. The data clearly indicate that iodoacetate is far more effective in potentiating the action of terbutaline than that of noradrenaline. The effect on the response to adrenaline is intermediate. In contrast to the actions of iodoacetate, pyruvate was a much stronger inhibitor of the response to noradrenaline than to terbutaline.

The molecular events causing these alterations in response have not been clarified. It does not seem likely that the modifications by iodoacetate and pyruvate of the adrenergic effect on phosphorylase could be explained by variation in the concentration of cyclic AMP. In a recent publication we have shown that while iodoacetate and fluoroacetate increase the adrenergic effect on phosphorylase, these agents decrease the levels of cyclic AMP attained following stimulation by adrenaline [2]. Pyruvate, which inhibits the response of the phosphorylase system to adrenergic agents, was without effect on the cyclic AMP response. The lack of correlation between cyclic AMP level, phosphorylase *b* kinase activation and conversion of phosphorylase *b* to *a* in muscle has been recently discussed by Gross and Mayer [15].

Sequestration of Ca^{2+} is an energy-dependent process and it is known that metabolic inhibitors can lead to Ca^{2+} release and contracture in skeletal muscle [16] and can also cause release of Ca^{2+} from isolated mitochondria [17]. It has been demonstrated that Ca^{2+} levels below those required to initiate contraction can promote formation of phosphorylase *a* through activation of phosphorylase *b* kinase independent of the action of cyclic AMP and protein kinase [5, 6, 18, 19]. In a recent publication we reported that phosphorylase conversion following tetanic contractions was even more potentiated by iodoacetate and more inhibited by pyruvate than was the stimulation by adrenaline [1]. Tetanic stimulation

of skeletal muscle has been shown to elicit conversion of phosphorylase *b* to *a* without phosphorylation of phosphorylase *b* kinase or increases in cyclic AMP concentration. Accordingly, it has been suggested that the Ca^{2+} released during tetanic contractions directly stimulates the catalytic activity of dephosphorylated phosphorylase *b* kinase resulting in increased formation of phosphorylase *a* [3, 20]. Based upon these observations we suggested that iodoacetate might increase the availability of Ca^{2+} for activation of phosphorylase *b* kinase while pyruvate, on the other hand, might reduce the Ca^{2+} activation of this enzyme by providing an excess of a readily oxidized substrate [1]. Several research groups have provided evidence that Ca^{2+} ions can act as second messengers [3, 5, 21–25]. Since, in the present study, it was shown that iodoacetate and pyruvate act quite differently on the stimulation of phosphorylase conversion by terbutaline and noradrenaline, the possibility exists that these agonists might act differently upon cytoplasmic Ca^{2+} distribution in muscle. It should, however, be pointed out that other factors might be involved as well in the modifications exerted by iodoacetate and pyruvate on the response of the phosphorylase system to adrenergic stimulation.

The suggestion that regulation of cytoplasmic Ca^{2+} levels may modulate enzyme activities has recently been made also by Meli and Bygrave [26]. These authors suggest that the mitochondria are involved in Ca^{2+} regulation of the activities of pyruvate kinase and other cytoplasmic enzymes.

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