Parallel metabotropic pathways in the heart of the toad, *Bufo marinus*

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**Bramich, Narelle J., Helen M. Cousins, F. R. Edwards, and G. D. S. Hirst.** Parallel metabotropic pathways in the heart of the toad, *Bufo marinus*. *Am J Physiol Heart Circ Physiol* 281: H1771–H1777, 2001.—This study examined the transduction pathways activated by epinephrine in the pacemaker region of the toad heart. Recordings of membrane potential, force, and intracellular Ca²⁺ concentration ([Ca²⁺]) were made from arrested toad sinus venosus. Sympathetic nerve stimulation activated non-α-, non-β-adrenoceptors to evoke a membrane depolarization and a transient increase in [Ca²⁺]. In contrast, the β-adrenoceptor agonist isoprenaline (10 μM) caused membrane hyperpolarization and decreased [Ca²⁺]. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (0.5 mM) mimicked the isoprenaline-evoked membrane hyperpolarization. Epinephrine (10–50 μM) caused an initial membrane depolarization and an increase in [Ca²⁺], followed by membrane hyperpolarization and decreased [Ca²⁺]. The membrane depolarizations evoked by sympathetic nerve stimulation or epinephrine were abolished either by the phospholipase C inhibitor U-73122 (20 μM) or by the blocker of D-myoo-inositol 1,4,5-trisphosphate-induced Ca²⁺ release, 2-aminoethoxydiphenyl borate (2-APB, 60 μM). Neither U-73122 nor 2-APB had an affect on the membrane hyperpolarization evoked by β-adrenoceptor activation. These results suggest that in the toad sinus venosus, two distinct transduction pathways can be activated by epinephrine to cause an increase in heart rate.

Cardiac; inositol trisphosphate; cAMP

**STIMULATION OF THE SYMPATHETIC nerves innervating mammalian and amphibian hearts causes an increase in heartbeat rate (12, 21). These responses are generally assumed to result from the activation of postjuncti-**

**nal β-adrenoceptors by catecholamines, with such β-adrenoceptors being linked to the activation of ade-**

**nylate cyclase and production of cAMP (13). However, catecholamines released from sympathetic nerves innervating amphibian and mammalian cardiac pacemaker tissue appear to combine with a distinct set of adrenoceptors linked to a pathway that does not in-**

**volve cAMP (4, 6, 8). In both mammals and amphibians, the adrenoceptors linked to the cAMP-independent pathway are likely to have a junctional location residing within the neuroeffector cleft and are stimulated readily by a neurally released transmitter. Exog-**

**enously applied transmitters do not readily activate**

junctional receptors but easily activate extrajunctional receptors that are located outside the neuroeffector cleft and are linked to the cAMP-dependent pathway (4, 6, 8). A comparison of the differences between neurally released and exogenously applied catecholamine is most easily studied in amphibians where, in contrast to mammals, the junctional and extrajunctional adre-**

**noceptors are pharmacologically distinct, junctional receptors being non-α-, non-β-adrenoceptors and extrajunctional receptors being β-adrenoceptors. In amphibians, the differences between the activation of the two types of adrenoceptors are most apparent in pacemaker preparations in which the discharge of pacem-**

**aker action potentials has been prevented by organic Ca²⁺ antagonists. In these arrested preparations, the activation of β-adrenoceptors causes membrane hyperpolarization as does the elevation of cAMP. In contrast, sympathetic nerve stimulation initiates a membrane depolarization that does not appear to involve the elevation of cAMP (4). The adrenoceptors activated either by a neurally released transmitter or high concentrations of rapidly applied epinephrine cannot be blocked with either α- or β-adrenoceptor antagonists (6, 19). However, such receptors can be blocked with dihydroergotamine (6). This indicates that both neuro-**

**nally released epinephrine and bath-applied epinephrine can activate a set of dihydroergotamine-sensitive receptors that are non-α-, non-β-adrenoceptors. When activated, these receptors cause the release of Ca²⁺ from intracellular stores and initiate positive chronotropic and inotropic responses (5). Furthermore, tran-**

**sient increases in intracellular Ca²⁺ concentration ([Ca²⁺]) accompany membrane depolarization evoked by stimulation of the junctional non-α-, non-β-adreno-**

**ceptrons in arrested preparations (9). In many other preparations, transmitter-evoked release of Ca²⁺ from intracellular Ca²⁺ stores is triggered by the production of D-myoo-inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] (2). First, this study examined the differences between the responses produced by β-adrenoceptor activation and those produced by sympathetic nerve stimulation and the activation of non-α-, non-β-adrenoceptors in ar-**

**rested preparations of toad sinus venosus. Second, the hypothesis was tested that, in the toad sinus venosus,**

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the stimulation of non-α-, non-β-adrenoceptors results in
the activation of a pathway involving the elevation of Ins(1,4,5)P3.

METHODS

The procedures described have been approved by the ani-
mal experimentation committee at the University of Mel-
bourne. Toads (Bufo marinus) were anesthetized by immor-
tion in a solution of 0.5% tricaine methanesulfonate
(Thomson and Joseph, Norwich, UK) in tap water. Prepara-
tions consisted of the sinus venosus and two atria, with the
ventricle and truncus arteriosus removed. Both the left and
right vagosympathetic trunks were dissected free back to the
sympathetic chains and left intact with the sinus venosus.
Preparations were pinned flat in a shallow recording cham-
ber, the base of which consisted of a microscope coverslip
coated with Sylgard silicone resin (9) and continuously per-
 fused with physiological saline solution containing the mem-
bbrane potential and relative changes in [Ca2+]i were made
from the sinus venosus, the pacemaker region of the toad heart. In the presence
of nifedipine (10 µM), the membrane potential was stable and lay in the range of
−31 to −39 mV, mean −35 ± 1 mV (n = 25 hearts).

In arrested preparations of toad sinus venosus,
trains of sympathetic nerve stimuli (10 impulses at 10 Hz)
certained a complex membrane depolarization that
typically displayed oscillations (Fig. 1A, trace a) (4, 6).
These depolarizations had amplitudes of 25.2 ± 2.1 mV
(n = 7) and were initiated after a latency (time elapsed
between the start of the stimulation train and the
attainment of 10% of the peak amplitude) of 1.27 ±
0.04 s. When the [Ca2+]i was monitored simulta-
neously, it was apparent that the depolarizations pro-
duced by sympathetic nerve stimulation were associ-
ated with an increase in [Ca2+]i (Fig. 1A, trace b) (5). Stimula-
tion with 10 impulses delivered at 10 Hz
evoked an increase in [Ca2+]i, with an amplitude of
0.15 ± 0.03 F340/380 (n = 5).

In contrast, in arrested preparations, the activation of
β-adrenoceptors evoked a membrane hyperpolariza-
 tion (Fig. 1B, trace a) (4). Rapid perfusion of isopro-
pramine (50 µM) over the sinus venosus for 30 s evoked a
membrane hyperpolarization that had a mean nadir of
3.5 ± 0.1 mV and lasted −5 min (n = 3). This mem-
bane hyperpolarization was associated with a de-
crease in [Ca2+]i of 0.12 ± 0.02 F340/380 (Fig. 1B, trace
b; n = 3) (10). This hyperpolarization was mimicked by
activating adenylate cyclase or by inhibiting phos-
phodiesterases (4). Thus the addition of the phospho-
diesterase inhibitor IBMX (0.5 mM) to the solution
bathing the sinus venosus evoked a membrane hyper-
polarization with a similar magnitude to that evoked
by β-adrenoceptor activation (Fig. 1C). The ionic basis
of the membrane potential change produced by iso-
pramlane or by IBMX detected in arrested amphibian
pacemaker cells remains unclear. However, it is clear
that β-adrenoceptor agonists and agents that cause
the elevation of cAMP produce similar sequences of
membrane potential changes (4, 13) but ones quite different
to those produced by sympathetic nerve stimulation.

The rapid perfusion of epinephrine (10 µM) onto the
arrested sinus venosus evoked an initial transient
membrane depolarization of 9.7 ± 1.2 mV followed by a
hyperpolarization with peak nadir of 3.3 ± 0.6 mV (n =

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ing that it resulted from β-adrenoceptor activation. In the presence of propranolol (1 μM), applied epinephrine evoked only a depolarization that was abolished by dihydroergotamine (10 μM; Fig. 2B, trace c) (6). This effect of dihydroergotamine was not due to its action as an α-adrenoceptor antagonist because membrane depolarizations persist in the presence of other α-adrenoceptor antagonists (6). These results suggest that the rapid bath application of epinephrine results in the activation of non-α-, non-β-adrenoceptors like that produced by sympathetic nerve stimulation as well as the activation of β-adrenoceptors (4, 6, 9).

Effect of phospholipase C inhibition on responses to epinephrine and sympathetic nerve stimulation in arrested sinus venosus. Previous experiments show that sympathetic nerve stimulation and applied isoprenaline initiate distinct sequences of membrane potential changes presumably by activating different metabolic pathways. Sympathetic nerve stimulation is associated with an increase in [Ca^{2+}]_i, resulting from Ca^{2+} store release that does not involve the activation of cAMP.
(4, 9). Very often Ca\(^{2+}\) store release results from the activation of an Ins(1,4,5)P\(_3\)-dependent pathway (2). The possibility that such a pathway mediated responses resulting from the activation of non-\(\alpha\)-, non-\(\beta\)-adrenoceptors was investigated by examining the effects of inhibiting phospholipase C (PLC) with U-73122 (3, 23) on responses evoked by sympathetic nerve stimulation. Responses evoked by sympathetic nerve stimulation (10 impulses at 10 Hz, \(n = 5\)) were abolished with U-73122 (20 \(\mu\)M). However, the effect of this agent may have resulted in part, if not entirely, from effects on transmitter secretion. In parallel sets of experiments, U-73122 (20 \(\mu\)M) abolished the responses to vagus nerve stimulation (\(n = 3\)). To avoid this complication, non-\(\alpha\), non-\(\beta\)-adrenoceptors were activated by the rapid application of epinephrine. Figure 3A, traces \(a\) and \(b\), shows that the PLC inhibitor U-73122 (20 \(\mu\)M) abolished the membrane depolarization produced by applied epinephrine (\(n = 5\)). However, the membrane hyperpolarization resulting from the activation of \(\beta\)-adrenoceptors was unchanged.

This indicates that U-73122 neither blocks \(\beta\)-adrenoceptors nor interferes with the cAMP-dependent pathway. The simplest explanation is that U-73122 inhibits the depolarization evoked by the activation of non-\(\alpha\), non-\(\beta\)-adrenoceptors by preventing PLC activation. As a further control, the effects of the closely related compound U-73343 (3) on the responses to applied epinephrine were examined. U-73343 (20 \(\mu\)M), which does not block PLC, had no effect on the amplitude of either the depolarization or the afterhyperpolarization produced by added epinephrine (\(P > 0.05, n = 5\); Fig. 3B, traces \(a\) and \(b\)).

In a number of tissues, it has been shown that, in addition to inhibiting PLC, U-73122 may also cause the release of Ca\(^{2+}\) from intracellular stores (16, 18). To examine whether the effects of U-73122 were due to Ca\(^{2+}\) store depletion, the effect of U-73122 on responses evoked by application of caffeine (10 mM) was examined. It has previously been shown (5) that the rapid bath application of high concentrations of caf-
feine causes a membrane depolarization that is oscillatory in nature (Fig. 4A, trace a). This is associated with an oscillatory increase in force production of the sinus venosus (Fig. 4A, trace b). These changes in membrane potential and force are due to the release of Ca\(^{2+}\) from ryanodine-sensitive stores (5). U-73122 (20 \(\mu\)M) had little effect on the underlying membrane depolarization or increase in force production evoked by caffeine; however, it abolished the oscillations in both membrane potential and force (Fig. 4B). Therefore, part of the effects of U-73122 on responses evoked by sympathetic nerve stimulation may have been due to its action on Ca\(^{2+}\) stores rather than PLC inhibition.

To further examine the possibility that Ins(1,4,5)P\(_3\) production was necessary for the response produced by the activation of non-\(\alpha\)-, non-\(\beta\)-adrenoceptors, the effects of inhibition of Ins(1,4,5)P\(_3\)-induced Ca\(^{2+}\) release by 2-APB (60 \(\mu\)M) (1, 17) were examined on both membrane potential and force changes evoked by sympathetic nerve stimulation. In control solution, stimulation of the sympathetic nerves evoked a membrane depolarization of 19 ± 5 mV \((n = 5)\). The addition of 2-APB (60 \(\mu\)M) to the physiological saline solution (30 min) had no effect on either the resting membrane potential or the basal force production. However, 2-APB significantly reduced the amplitude of the nerve-evoked membrane depolarization \((4 \pm 2 \text{ mV}, P = 0.01, n = 5, \text{Fig. 5A})\). Washout of 2-APB for 60 min partially restored the response to sympathetic nerve stimulation \((15 \pm 3 \text{ mV})\). In four experiments, the effect of 2-APB (60 \(\mu\)M) on force changes evoked by sympathetic nerve stimulation was also examined. Before the addition of 2-APB, sympathetic nerve stimulation evoked an increase in basal force of 0.28 ± 0.10 mN. After the addition of 2-APB, the increase in force production evoked by sympathetic nerve stimulation was significantly reduced \((0.03 \pm 0.01 \text{ mN}; P = 0.02)\). At 60 \(\mu\)M, 2-APB had no effect on responses evoked by vagus nerve stimulation \((n = 3)\), suggesting that it was not inhibiting transmitter release.

The effect of 2-APB was also examined on responses evoked by the bath application of epinephrine. If
2-APB was acting specifically to inhibit Ins(1,4,5)P$_3$-induced Ca$^{2+}$ release, it would be expected that 2-APB would abolish the membrane depolarization resulting from the activation of non-α-, non-β-adrenoceptors but have no effect on the membrane hyperpolarization resulting from β-adrenoceptor activation. Epinephrine (50 µM) evoked an initial membrane depolarization of 7 ± 2 mV followed by a membrane hyperpolarization of 4 ± 1 mV (n = 4) in amplitude (Fig. 5B, trace a). The addition of 2-APB (60 µM, 30 min) significantly reduced the amplitude of the membrane depolarization (1 ± 1 mV; P = 0.04) but had no effect on the membrane hyperpolarization (5 ± 1 mV; P = 0.09; n = 4; Fig. 5B, trace b).

Although 2-APB (60 µM) has previously been shown to have no effect on either the production of cAMP or Ca$^{2+}$-induced Ca$^{2+}$ release, 2-APB has been shown to partially inhibit the reuptake of Ca$^{2+}$ into Ins(1,4,5)P$_3$-sensitive Ca$^{2+}$ stores when used at high concentrations (>100 µM) (17). To examine whether 2-APB was depleting intracellular stores due to the inhibition of Ca$^{2+}$ reuptake, the effect of 2-APB on changes in membrane potential and force evoked by caffeine was examined. Both the membrane depolarization and increase in force evoked by caffeine persisted in the presence of 2-APB (60 µM, n = 6, Fig. 6). In addition, both the oscillations in membrane potential and force production also persisted in the presence of 2-APB.

**DISCUSSION**

This study suggests that two distinct transduction pathways, one involving cAMP and the other involving Ins(1,4,5)P$_3$, are present and can be activated by catecholamines in the toad sinus venosus. In the toad sinus venosus, just as in mammalian pacemaker preparations, applied catecholamines most readily activate β-adrenoceptors to elevate cAMP. This pathway results in the phosphorylation-dependent and -independent modulation of voltage-dependent channels involved in pacemaking activity (11, 13). The resulting tachycardia is associated with an increase in the amplitude of both pacemaker action potentials (4, 6) and the [Ca$^{2+}$]i transient that accompanies each action potential (5).

In contrast, responses evoked by sympathetic nerve stimulation resulting from activation of non-α-, non-β-adrenoceptors are not associated with an increased influx of Ca$^{2+}$ through voltage-dependent Ca$^{2+}$ channels (5) and do not involve cAMP (4). However, such responses are likely to involve a different complex metabotropic pathway; both the sympathetic tachycardias recorded from beating preparations and the membrane depolarizations recorded in arrested preparations begin after a long latency and last many seconds even when triggered by only a few sympathetic stimuli (4, 9). In arrested preparations, stimulation of the sympathetic nerves evokes an oscillatory membrane depolarization and a concomitant increase in [Ca$^{2+}$]i (9), both of which have temporal characteristics similar to the sympathetic tachycardia recorded from beating preparations (6). This suggests that a similar transduction pathway is activated in beating and arrested preparations of toad sinus venosus. We have recently shown that both the membrane depolarization and the increase in [Ca$^{2+}$]i evoked by sympathetic nerve stimulation in arrested preparations result from the release of intracellularly stored Ca$^{2+}$ (9). Similar responses also resulting from the activation of non-α-, non-β-adrenoceptors can be produced by rapid bath application of epinephrine to the arrested pacemaker tissue (Fig. 2) (4, 6). Responses evoked by the activation of these receptors were inhibited by prevention of Ins(1,4,5)P$_3$-induced Ca$^{2+}$ release. This was achieved by either preventing the formation of Ins(1,4,5)P$_3$ with the PLC inhibitor U-73122 or by directly preventing Ins(1,4,5)P$_3$-induced Ca$^{2+}$ release with 2-APB (Figs. 3 and 5). This suggests that the responses involve the activation of PLC and the formation of Ins(1,4,5)P$_3$, which triggers the release of Ca$^{2+}$ from intracellular stores. The subsequent activation of the Na$^+$/Ca$^{2+}$ exchanger found in cardiac muscle (7, 14) would provide a net inward current, which could account for both the depolarization in arrested preparations and the tachycardia in beating preparations (15).

How these findings relate to mammalian pacemaker cells remains unclear. In the mammalian heart, both neurally released and added norepinephrine activate β-adrenoceptors. However, the responses to neurally released and added norepinephrine differ in much the same way as they do in the amphibian heart (8). Clearly, one explanation could be that the β-adrenoceptors under sympathetic nerve terminals are linked to PLC, whereas those at a more distant location are linked to adenylate cyclase. However, we know of no example where a β-adrenoceptor is linked to PLC. Indeed in mammalian hearts, this pathway is usually linked to an α-adrenoceptor (20, 22).

Whatever the case, it is clear that in both amphibians and mammals, the same transmitter activates separate metabotropic pathways. One pathway is activated by the local high concentrations of transmitter achieved after nerve activity. In amphibians, this pathway appears to involve the second messenger Ins(1,4,5)P$_3$, which releases Ca$^{2+}$ from an intracellular store to increase heart rate. The other pathway, which involves cAMP, is activated by a more diffuse action of transmitter such as that occurring during a rise in the concentration of catecholamine circulating in the blood.

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