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SHORT COMMUNICATION

GLIBENCLAMIDE BINDING IN VERTEBRATE CARDIAC MEMBRANES

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ATP-sensitive potassium channels (K_{ATP}) were discovered by Noma (1983) in the cardiac myocyte. These channels are modulated by intracellular ATP concentration. They are usually open and leak potassium under conditions when cellular energy demand exceeds energy supply, for example during hypoxia or ischemia. They are also found in the central nervous system, smooth muscle and pancreas (Ashcroft, 1988). Their role in the control of insulin secretion has been well established (Petersen, 1990). Recent studies have shown that K_{ATP} channels are important in protecting the heart during ischemia (Grover *et al.* 1989; Gross and Auchampach, 1992) and during hypoxia (McKean and Branz, 1992). These channels have also been implicated in the phenomenon of preconditioning (Auchampach *et al.* 1992). The mechanism for cardiac protection has not been well established; however, K_{ATP} opening shortens the cardiac action potential and may limit calcium entry and reduce the metabolic demand of the myocyte (Cole *et al.* 1991). The channel opening is responsible for an initial extracellular accumulation of potassium that may also serve to decrease metabolic demand on the heart (Wilde *et al.* 1990). The open channels, however, could be detrimental to the heart during reperfusion or reoxygenation as they would facilitate re-entry phenomena and arrhythmias (Wolleben *et al.* 1989). Studies from this laboratory have shown that the heart of the semi-aquatic diving rodent, the muskrat (*Ondatra zibethicus*), is able to survive ischemia and hypoxia better than the heart of a similarly sized non-diving rodent, the guinea pig (McKean and Landon, 1982; McKean, 1984). We hypothesized that the K_{ATP} channel might be involved in the explanation for this species difference.

The purpose of this study was to perform saturation binding studies on the K_{ATP} channels in several rodent species to determine whether the binding properties (β_{max} and K_d) of the channels in the heart from a hypoxia-adapted animal differ from those found in common laboratory rodents. The radioligand used was glibenclamide, a sulfonylurea and a specific K_{ATP} channel antagonist (Miller *et al.* 1991). Since little is known of the distribution of these channels in the hearts of 'lower' vertebrates, a second purpose of this study was to examine saturation binding of glibenclamide to cardiac membranes prepared

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from a wide variety of vertebrate hearts. In some vertebrate classes, species having different tolerances to hypoxia were included.

Membrane suspensions were prepared from a crude cardiac homogenate (Gopalakrishnan *et al.* 1991). No attempt was made to purify the preparation because tissue samples from the different species may purify differently, rendering a quantitative comparison difficult to interpret. Hearts were generally removed from ether-anesthetized animals and were immediately flushed with 20ml of an ice-cold saline solution. Fish hearts were removed after the animals had been stunned by a sharp blow to the head. Rattlesnakes were chilled in ice and then decapitated prior to removal of the heart. Several grams of tissue were minced and suspended in 15ml of ice-cold 50mmol l^{-1} Tris buffer, pH7.2. The suspension was homogenized with a polytron homogenizer twice for 15s at a setting of 6.5. Large 'strings' of connective tissue were removed by filtration through organdy fabric. The homogenate was centrifuged for 45 min at 48 000 ***g***. The supernatant was discarded and the pellet resuspended in 15ml of Tris buffer by polytron homogenization for 15s at a speed of 5 followed by three strokes of a Potter–Elvehjem homogenizer at a speed of 4. The homogenate was placed on ice for 1min between each of the homogenization steps. The final homogenate was placed on ice and continuously stirred with a magnetic stirrer. Protein content in vesicles treated with 1% Triton X-100 was determined using the BioRad DC assay (BioRad, Richmond, CA) with bovine serum albumin as the protein standard. Saturation binding was performed in duplicate on 0.5mg of membranes in a total volume of 1.05 ml. [^{3}H]glybenclamide (specific activity 50.9 Cimmol$^{-1,}$ DuPont NEN, Boston, MA) was added over a concentration range of 10^{-11}–10^{-8} mol l^{-1}. Non-specific binding was determined in tubes to which 10^{-6} mol l^{-1} unlabeled glibenclamide had been added. The tubes were vortex-mixed, covered and allowed to incubate at 20°C for 1h. Membrane preparations were filtered using a cell harvester and GF-B glassfiber filters. Filtered membranes were rinsed three times with 5ml of ice-cold 50 mmol l^{-1} Tris buffer and placed in a biodegradable liquid scintillation cocktail for counting. Total binding was defined as the binding of ^{3}H ligand in the absence of unlabeled glibenclamide. Specific binding was taken as the difference between total binding and binding in the presence of unlabeled glibenclamide. Only ventricular tissue was used unless otherwise indicated.

Fig. 1 shows the total and specific binding of glibenclamide to membranes derived from muskrat atria. The specific binding is saturable and Scatchard analysis (Fig. 2) revealed that the data are consistent with binding to a single site. Fig. 3 shows glibenclamide binding in membranes derived from guinea pig ventricle. Specific binding could not be demonstrated in cardiac membrane preparations obtained from this and nine other guinea pigs. The density of specific glibenclamide binding (β_{max}) in muskrat heart membranes (six ventricles, one atrium) was 79.3±48.4fmolmg^{-1} compared with 38.4±20.1fmolmg^{-1} in rat heart membranes (six ventricles, two atria). These values are significantly different (Student's *t*-test, P=0.047). The K_d value for muskrat heart membranes was 2.75±2.64nmol l^{-1} while the K_d for rat was 1.51±1.31nmol l^{-1}. These values are not significantly different (P=0.233). A systematic study of glibenclamide binding in atria *versus* ventricle was not carried out; however, there did not appear to be

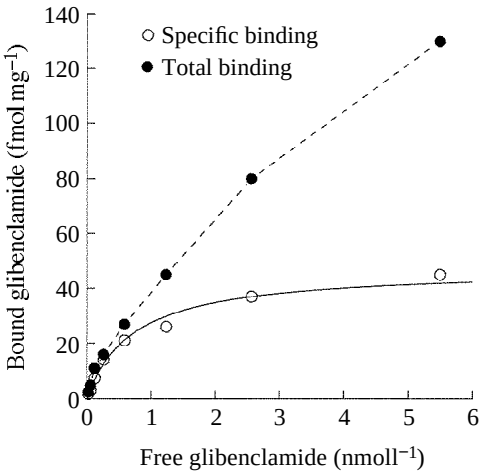

Fig. 1. [^{3}H]glibenclamide binding in muskrat atria. The atria from three muskrat hearts were combined and homogenized to make the membrane preparation. Specific and non-specific binding were determined as described in the text.

major differences in the few preparations that were examined. Therefore, the atrial data were combined with the data obtained from ventricles.

Failure to demonstrate specific binding in guinea pig heart is probably due either to the relatively low number or to the low affinity of these binding sites. We were able to detect specific binding from guinea pig brain membrane preparations using the same methodology. Fosset *et al.* (1988) measured specific binding in purified microsomes obtained from guinea pig ventricles. The β_{max} for guinea pig was about 65% of the value for the chicken ventricle and the K_d value was six times higher (lower affinity). If we had

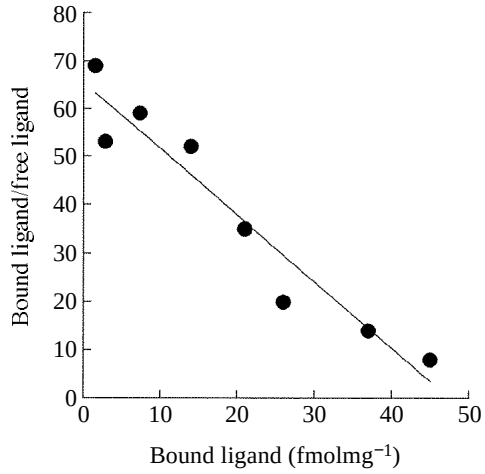

Fig. 2. Scatchard plot of muskrat atrium saturation binding data. Data are from the membrane preparation shown in Fig. 1. The line is a linear regression fitted to the data points (r^2=0.962).

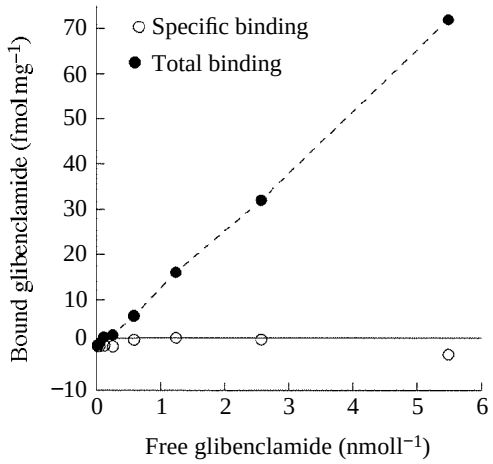

Fig. 3. Saturation binding data for guinea pig left ventricle.

chosen to purify the membrane preparations, it is likely that we could have determined β_{max} and K_d values in the guinea pig heart. Purification was not attempted, however, because the hearts from different species would probably purify differently and thus species comparisons would have been difficult to interpret. Even though it was not possible to establish the presence of K_{ATP} channels in guinea pig hearts through glibenclamide binding studies, their activity has been demonstrated in this species using physiological (Weiss and Lamp, 1989) and pharmacological (McKean and Branz, 1992) methods.

Glibenclamide binding was also determined in (left) ventricular homogenates from different classes of vertebrates (Table 1). K_{ATP} channels were detected by radioligand binding in the amphibians, but specific binding was too low for detection in the agnathan and teleost fishes. Small numbers of fishes were used in the study as detectable binding in membrane preparations was not evident after studing one or two animals and increasing the sample size served no useful purpose. Cane toads (*Bufo marinus*) had a channel density (β_{max}) approximately twice that of the leopard frog (*Rana pipiens*); affinities (K_d) were similar. Both of these species are tolerant of hypoxia compared with mammals. Leopard frogs are semi-aquatic and can stay submerged or buried in mud for many hours. The cane toad is frequently found in brackish water and probably encounters a hypoxic environment in burrows (Pörtner *et al.* 1991). Both of the reptiles studied had high densities of specific glibenclamide binding. The turtle (*Chrysemys picta*) is an example of a diving vertebrate and its brain tissue has been shown to be hypoxia-tolerent (Lutz, 1992), yet glibenclamide binding in the adult turtle brain is negligible compared with rat brain binding (Xia and Haddad, 1991). The rattlesnake (*Crotalus viridus*) has a terrestrial habit, but may encounter a hypoxic environment in its den or during prey capture in burrows. The pigeon (*Columba* sp.) was the sole representative of the class Aves in the study and is not likely to have developed adaptations to hypoxia. The birds used in this study resided at 75m elevation and did not fly at great altitude, dive under water or

Table 1. *Glibenclamide binding in vertebrate left ventricles*

Species	Number of animals	Number of experiments	β_{max} (fmol mg^{-1})	K_d (nmol l^{-1})
Lamprey	6	3	NDB	
Chinook salmon	2	2	NDB	
Carp	1	1	NDB	
Cane toad	7	5	450.3±325.0	30.3±22.0
Leopard frog	20	3	267.3±154.2	45.3±31.3
Painted turtle	6	3	443.3±157.1	7.4±4.3
Rattlesnake	5	3	349.3±42.8	2.50±2.2
Pigeon	3	3	272.0±31.7	11.7±11.7
Guinea pig	10	10	NDB	
Rat	6	6	33.9±21.8	1.5±1.5
Muskrat	6	6	81.8±52.5	6.8±9.5

NDB, no detectable binding.
Values are given as mean ± S.D.

burrow. In spite of their habitat, the pigeons had a fairly high density of specific glibenclamide binding with 10^{-8} mol l^{-1} affinity for antagonist.

Opening of the K_{ATP} channel causes a shortening of the cardiac action potential (Findlay *et al.* 1989) and the time available for calcium influx. This would reduce the strength of contraction. Channel opening also causes a cell hyperpolarization, making the cell less excitable. With continued opening, there is an accumulation of extracellular potassium, which ultimately depolarizes the cell, rendering it inexcitable because of sodium channel inactivation. All of these mechanisms would have a cardioprotective effect during metabolic stress by conserving the amount of ATP used in the mechanical activity of the heart. In the atria, the channels might serve a different function since mechanical activity at this site is not an important energy consumer in comparison with the ventricle. Channel opening in the atria might serve to decrease heart rate and thus to provide energy savings by decreasing the rate at which the heart pumps. Heart rate in isolated denervated hearts is known to decrease during hypoxia and it is possible that K_{ATP} channels may participate in the initiation and maintenance of this response. The bradycardia of hypoxia is much more pronounced in muskrat hearts than in guinea pig hearts (McKean and Landon, 1982) and this is consistent with the muskrat atrium having higher glibenclamide binding than the guinea pig atrium. A direct comparison of these species with rat heart has not been made. Although much is known about the physiology of the K_{ATP} channel in the heart, the function of this channel is much less clear. Reptiles, birds and amphibians have cardiac membranes which specifically bind glibenclamide to a high degree. It is not known if this binding is to functional K_{ATP} channels that behave in a similar fashion to mammalian K_{ATP} channels. Additional experiments are needed to clarify this issue. The function of these channels is not known in these vertebrate classes. A protective role of the K_{ATP} channel during hypoxia has been demonstrated in the guinea pig heart, even though the density of these channels was sufficiently low to escape detection using the binding methodology in this study. Unpublished observations from this laboratory indicate that

muskrat hearts, which have a relatively high density of specific glibenclamide binding, do not survive hypoxic perfusion when the K_{ATP} channels are blocked with the channel antagonist, glibenclamide. Hypoxic muskrat hearts almost immmediately develop irreversible arrhythmias and contracture. Compared with the muskrat heart, avian, reptilian and amphibian hearts bind approximately 4–6 times more K_{ATP} channel ligand. Perhaps channel activation differs among these species but, nonetheless, it is interesting that these species appear to have a very high channel density. Although the reptiles and amphibians may be adapted to hypoxia and the channels may, in some fashion, be involved with hypoxia tolerance, the pigeon heart also has large numbers of channels; their function in pigeons is not immediately obvious. Knowledge of the channel density in a hypoxia-adapted bird such as a diving bird might help to clarify this issue.

The K_{ATP} channel antagonist binding properties of crude membranes obtained from guinea pig, rat and muskrat hearts have been determined. The density is greatest in the heart of the hypoxia-adapted muskrat and least in the guinea pig. Specific binding of this K_{ATP} channel antagonist was not detectable in agnathan or teleost hearts, but was of large magnitude in amphibia, reptiles and bird hearts.

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