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AMP kinase regulates ligand-gated K-ATP channels in substantia nigra dopamine neurons

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Abstract

AMP-activated protein kinase (AMPK) is a master enzyme that regulates ATP-sensitive K⁺ (K-ATP) channels in pancreatic beta-cells and cardiac myocytes. We used patch pipettes to record currents and potentials to investigate effects of AMPK on K-ATP currents in substantia nigra compacta (SNC) dopamine neurons in slices of rat midbrain. When slices were superfused repeatedly with the K-ATP channel opener diazoxide, we were surprised to find that diazoxide currents gradually increased in magnitude, reaching 300% of the control value 60 min after starting whole-cell recording. However, diazoxide current increased significantly more, to 472% of control, when recorded in the presence of the AMPK activator A769662. Moreover, superfusing the slice with the AMPK blocking agent dorsomorphin significantly reduced diazoxide current to 38% of control. Control experiments showed that outward currents evoked by the K-ATP channel opener NN-414 also increased over time, but not currents evoked by the GABA_B agonist baclofen. Delaying the application of diazoxide after starting whole-cell recording correlated with augmentation of current. Loose-patch recording showed that diazoxide produced a 34% slowing of spontaneous firing rate that did not intensify with repeated applications of diazoxide. However, superfusion with A769662 significantly augmented the inhibitory effect of diazoxide on firing rate. We conclude that K-ATP channel function is augmented by AMPK, which is activated during the process of making whole-cell recordings. Our results suggest that AMPK and K-ATP interactions may play an important role in regulating dopamine neuronal excitability.

Keywords

AMP kinase; diazoxide; substantia nigra; brain slice; K-ATP; patch-clamp

INTRODUCTION

AMPK is a complex enzyme composed of an alpha catalytic subunit and beta and gamma regulatory subunits (Hardie et al., 2003). Widely expressed in brain and somatic tissues,

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AMPK is activated by rising levels of AMP that displaces ATP at the gamma subunit, which produces a conformational change that enables phosphorylation of the alpha subunit at Thr-172 (Oakhill et al., 2010). Once AMPK is bound by AMP, the upstream kinases liver kinase B1 (LKB1) and calcium-calmodulin-dependent kinase kinase beta (CaM-KK β) phosphorylate AMPK and cause AMPK activity to increase many fold (Amato and Man, 2011). In peripheral tissues such as liver and skeletal muscle, AMPK encourages processes that increase ATP production, such as increasing glucose uptake, accelerating glycolysis, and increasing the oxidation of fatty acids. Furthermore, AMPK inhibits pathways that consume ATP, such as the syntheses of fatty acids, glycogen and proteins. (Russo et al., 2013). Conditions that deplete energy stores, such as hypoglycemia, hypoxia, ischemia and calorie restriction, also activate AMPK (Hardie et al., 2003; Chiba et al., 2010). Thus, AMPK plays an important role in the conservation of cellular energy and in replenishing the supply of high-energy compounds.

AMPK is often linked to ATP-sensitive K⁺ (K-ATP) channels because both respond to alterations in metabolic conditions (Yoshida et al., 2012; Nichols, 2006). The K-ATP channel is a hetero-octameric complex composed of a sulfonylurea binding protein (SUR) and an inwardly rectifying K⁺ channel (Kir) (Nichols, 2006). K-ATP channels are blocked by rising ATP levels and are opened with falling energy levels. In pancreatic beta-cells, AMPK increases the expression of K-ATP channels on the cell surface and thereby facilitates insulin secretion (Lim et al., 2009; Smith et al., 2006). AMPK has also been shown to promote K-ATP channel opening in pancreas and cardiac myocytes (Yoshida et al., 2012; Beall et al., 2013). K-ATP channels in pancreas are composed of SUR1 and Kir6.2 subunits, which are also widely expressed in brain (Aguilar-Bryan and Bryan, 1999). Although AMPK and K-ATP channels are widely expressed in the brain, little is known of their interactions. Recently, our lab reported that K-ATP current could be generated by N-methyl-D-aspartate (NMDA) receptor stimulation through a Ca²⁺-dependent activation of AMPK in subthalamic nucleus (STN) neurons (Shen et al., 2014). This finding is important because the inhibitory influence of K-ATP current may tend to counterbalance the excitatory influence of glutamate receptor stimulation. Moreover, others have shown that K-ATP currents can modulate the firing pattern in a subset of dopamine neurons in rodent midbrain (Schiemann et al., 2012). These results suggest that K-ATP channels and their regulation by AMPK may be important for altering neuronal excitability and firing pattern.

Dopamine neurons in the substantia nigra zona compacta (SNC) play vital roles in goal-directed behaviors and behavioral reinforcement as well as in enabling normal movement. Because firing pattern is important in modifying dopamine release (Grace et al., 2007; Paladini and Roeper, 2014), a better understanding of how ion channels and membrane properties modify neuronal excitability could lead to better control of dopamine-dependent behaviors. The purpose of the present study was to characterize AMPK and K-ATP channel interactions in SNC dopamine neurons. Using patch-pipette recordings in slices of rat midbrain, we found that AMPK activation during whole-cell recordings cause gradual augmentation of ligand-gated K-ATP currents. Moreover, inhibition of AMPK activity caused rundown of K-ATP currents, suggesting that AMPK activity is necessary for normal K-ATP function. Our results suggest that an AMPK and K-ATP interaction may play an important role in regulating dopamine neuronal excitability.

EXPERIMENTAL PROCEDURES

All procedures were approved by the Institutional Animal Care and Use Committee at the Veterans Affairs Portland Health Care System. Every precaution was taken to minimize animal stress and the number of animals used. Tissue preparation and whole-cell recordings were done as reported previously by our lab (Shen et al., 2014).

Tissue Preparation

A total of 96 male Sprague-Dawley rats (80–180 g, Harlan, Indianapolis, IN) were used in our studies. Briefly, rats were anesthetized with isoflurane and euthanized by severing major thoracic vessels. Brains were rapidly removed and horizontal slices were cut with a vibratome (200–300 μm thick) in an ice-cold sucrose buffer solution of the following composition (in mM): sucrose, 196; KCl, 2.5; MgCl_2 , 3.5; CaCl_2 , 0.5; NaH_2PO_4 , 1.2; glucose, 20; and NaHCO_3 , 26, equilibrated with 95% O_2 and 5% CO_2 . A slice containing the SNC was then placed on a supporting net and submerged in a continuously flowing solution (2 ml/min) of the following composition (in mM): NaCl, 126; KCl, 2.5; CaCl_2 , 2.4; MgCl_2 , 1.2; NaH_2PO_4 , 1.2; NaHCO_3 , 19; glucose, 11, gassed with 95% O_2 and 5% CO_2 (pH 7.4) at 36°C. A low-power microscope was used to identify the SNC as gray matter rostral and caudal to the medial terminal nucleus of the accessory optic tract.

Electrophysiological Recordings

Whole-cell recordings were made with pipettes containing (in mM): potassium gluconate, 138; MgCl_2 , 2; CaCl_2 , 1; EGTA, 11; HEPES, 10; ATP, 1.5; GTP, 0.3 (pH 7.3). Pipette resistance ranged from 3 to 8 $\text{M}\Omega$, whereas series resistance typically ranged from 15 to 40 $\text{M}\Omega$. In some experiments, we used a “loose-patch” method to record extracellular potentials (Nunemaker et al., 2003). For loose-patch recordings, patch pipettes were filled with standard extracellular solution; patch resistance was typically 5–10 times the starting value. Most recordings were performed with the “blind” patch method in which slices were visualized with a dissection microscope (Leica StereoZoom-7, IL, USA) and whole-cell tight-seal recordings were made without visualization of individual neurons and without any cleaning of the slice surface (Blanton et al., 1989). Visualized patch recordings utilized an upright Zeiss Axioskop (CA, USA) microscope equipped with infra-red Nomarski optics to allow for visualization of individual neurons (Stuart et al., 1993). Membrane currents or potentials were amplified and recorded with an Axopatch-1D amplifier with 5-kHz low-pass filter (Molecular Devices, Foster City, CA, USA). Data were acquired using a personal computer with a Digidata analog/digital interface and analyzed using pCLAMP 10 software (Molecular Devices/Axon Instruments, Foster City, CA). Dopamine neurons were identified by their broad action potentials or currents and by inhibitory effects of dopamine (30 μM) on membrane potential or current. Membrane potentials for whole-cell recordings were corrected for the liquid junction potential (10 mV).

Current-Voltage Studies

Current-voltage (I-V) relationships were constructed by measuring currents during a series of hyperpolarizing voltage steps (400 ms duration) with 10 mV increments from a holding potential of -70 mV. Currents were measured immediately after capacitive transients to

minimize the influence of hyperpolarization-activated cation currents. When measuring the voltage dependence of diazoxide currents, I-V plots were always obtained at the end of the 5 min application of diazoxide. I-V plots are displayed as “subtracted” currents in which currents recorded during the experimental treatment were subtracted from those currents recorded in the control condition. Therefore, these subtracted currents represent “net” currents that were produced or blocked by an experimental treatment. Slope conductance was calculated for each cell as the slope of a straight line in the I-V plot at voltages between -80 and -120 mV. Mean slope conductance and S.E.M. were obtained by averaging slope conductance from all cells in each experimental group.

Drugs and Chemicals

All drugs were dissolved in aqueous or dimethyl sulfoxide stock solutions. Most drugs were added to the slice superfusate. Stock solutions were diluted at least 1:1000 to the desired concentration in superfusate immediately prior to use. Approximately 30 seconds were required for the drug solution to enter the recording chamber; this delay was due to passage of the superfusate through a heat exchanger. In some experiments, drugs were added to the internal pipette solution and delivered by passive intracellular dialysis. Diazoxide, dorsomorphin (Compound C), and 6,7-dihydro-4-hydroxy-3-(2'-hydroxy[1,1'-biphenyl]-4-yl)-6-oxo-thieno[2,3-b]pyridine-5-carbonitrile (A769662) were obtained from Tocris Cookson Inc. (Bristol, UK). Tolbutamide, NN-414, baclofen and dopamine HCl were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Data Analysis

Numerical data in the text and error bars in figures are expressed as mean $\pm$ S.E.M. Statistical tests were performed on a personal computer using IBM SPSS Statistics version 22 (IBM North America, New York, NY, USA). Outcome distributions were formally tested and data were log transformed when necessary in order to satisfy normality requirements. Significance was accepted with $P < 0.05$.

RESULTS

Effect of AMPK activation on diazoxide currents

Slices were superfused with diazoxide ($200 \mu\text{M}$) for 5 min every 30 min while recording SNC dopamine neurons in whole-cell configuration with patch pipettes that contained normal internal solution. Unexpectedly, we found that repeated applications of diazoxide evoked outward currents that progressively increased over time (Fig. 1A). This figure also shows that diazoxide current was strongly inhibited by the K-ATP inhibitor tolbutamide ($100 \mu\text{M}$). Averaged data in Fig. 1B show that diazoxide-induced currents increased significantly over time ($F_{(3,51)} = 7.42$, one-way ANOVA, $P < 0.01$). Under control conditions, the initial diazoxide current of 45 ± 7 pA ($n = 16$) increased to 113 ± 14 pA ($n = 15$) when measured 60 min later ($P < 0.01$, Sidak pairwise test). But despite this augmentation of diazoxide current under control conditions, we found that exposure to the AMPK activator A769662 caused a further increase in diazoxide currents. In these experiments, A769662 was either added to the pipette solution ($5 \mu\text{M}$) or added to the superfusate at least 30 min prior to recordings ($30 \mu\text{M}$). In the presence of A769662, the initial diazoxide current (33 ± 6 pA, n

= 13) increased to 189 ± 45 pA ($n = 7$) when measured 90 min later ($P < 0.001$, t test). Moreover, current recorded with A769662 at 90 min was significantly greater than that recorded under control conditions (114 ± 27 pA, $n = 8$; $P < 0.05$, one-tailed t test). Figure 1C shows normalized data for diazoxide-induced currents recorded with A769662, control internal solution, and in the presence of the AMPK inhibitor dorsomorphin (30 μ M). Superfusion with dorsomorphin began 5 min before the initial diazoxide application. There was a significant effect of treatment group on diazoxide currents over time ($F_{(2,80)} = 72.19$, two-way ANOVA, $P < 0.001$). Initial diazoxide currents recorded in A769662 (33 ± 6 pA; $n = 13$) and dorsomorphin (72 ± 19 pA; $n = 8$) were not significantly different from control (45 ± 7 pA; $n = 16$). Our previous Western immunoblot analysis confirmed that A769662 increased whereas dorsomorphin decreased AMPK Thr-172 phosphorylation in slices of rat midbrain and diencephalon (Shen et al., 2014). These data support the hypothesis that AMPK activation potentiates K-ATP channel function. Moreover, results also suggest that inhibition of AMPK activity reduces K-ATP channel function.

In a separate series of experiments we examined the effects of repeated applications of tolbutamide (100 μ M) to assess resting levels of K-ATP current. Five minutes after beginning whole-cell recording, a 5 min superfusion with tolbutamide produced no significant inward current (2 ± 2 pA, $n = 11$). However, a larger inward current (19 ± 12 pA, $n = 7$) was evoked by tolbutamide when measured 40 min after starting recording ($P < 0.05$, one-tailed t test). These results agree with those of Avshalumov et al (2005) who demonstrated a small but physiologically significant depolarizing influence of a K-ATP blocking agent on SNC dopamine neurons. Our results suggest that a K-ATP channel conductance may be present at rest and may increase slowly over the duration of recording in whole-cell configuration.

Diazoxide conductance increases over time

Whole-cell chord conductance was measured in order to confirm that diazoxide increases a K^+ conductance. Slices were superfused with diazoxide for 5 min with an interval of 20 min, and membrane conductance was calculated by measuring currents during a series of hyperpolarizing voltage steps. Recordings were performed using pipettes filled with normal internal solution. As shown in the current traces in Fig. 2A, the second application of diazoxide (200 μ M) produced a much larger increase in conductance compared to the first application. Averaged data for 5 neurons are shown in Fig. 2B. Conductance increase produced by the second diazoxide application (5.39 ± 0.78 nS) was significantly greater than that produced by the first application (2.32 ± 0.25 nS; $P < 0.01$, paired t test). Estimated reversal potentials for currents during the first diazoxide application (-103 ± 3 mV) and the second application (-100 ± 1 mV) were close to that expected for the K^+ reversal potential (-106 mV) as calculated by the Nernst equation.

Currents evoked by NN-414 also increase over time

In order to evaluate a K-ATP agonist other than diazoxide, we proceeded to measure currents evoked by the K-ATP channel opener NN-414. However, preliminary experiments showed that outward currents evoked by NN-414 were long-lasting and did not easily wash out over time. Therefore, we employed a procedure by which K-ATP currents were measured by

repeatedly applying the sulfonylurea agent tolbutamide in the continuous presence of NN-414. Thus, slices were superfused with tolbutamide (100 μ M) for 5 min every 20 min in the continuous presence of NN-414 (10 μ M), and K-ATP currents were measured as current blocked by tolbutamide. The current trace in Fig. 3A shows that tolbutamide-sensitive current gradually increased over time in the presence of NN-414. These data are summarized in Fig. 3B. There was a significant increase in tolbutamide-sensitive current in NN-414 over time ($F_{(3,3)} = 23.75$, $P < 0.001$, one-way repeated measures ANOVA). These data show that augmentation of ligand-gated K-ATP currents over time is not unique to diazoxide.

A769662 does not augment baclofen currents

We proceeded to investigate the effect of AMPK activation on currents evoked by the GABA_B agonist baclofen to see if augmentation of K-ATP current was generalizable to other K⁺ currents. Figure 4A shows a current trace from a dopamine neuron recorded with a pipette containing 5 μ M A769662. Currents evoked by bath application of baclofen (1 μ M) showed a gradual reduction in amplitude despite the presence of A769662. Averaged data in Fig. 4B show that baclofen-induced current declined from an initial value of 110 ± 8 pA to 86 ± 15 pA as measured 45 min later. These data show that the AMPK activator A769662 does not augment K⁺ current evoked by a GABA_B receptor agonist.

Effects of prior diazoxide exposure and duration of whole-cell recording

In order to explore the possibility that a sensitization phenomenon underlies potentiation of diazoxide currents, we tested the hypothesis that augmentation of diazoxide currents is dependent upon duration of neuronal exposure to diazoxide. We recorded effects of repeated applications of diazoxide in pairs of neurons that were recorded sequentially in the same brain slice. As expected, Fig. 5A shows significant augmentation of diazoxide currents in the first recorded neurons when tested repeatedly over one hour ($F_{(3,16)} = 7.375$, $P < 0.01$, one-way ANOVA). Furthermore, Fig. 5A shows that the second recorded neurons in the slice also showed significant augmentation of diazoxide current over time ($F_{(3,28)} = 4.804$, $P < 0.01$, one-way ANOVA). Importantly, we found that the initial diazoxide currents recorded in first (38 ± 8 pA) and second (32 ± 4 pA) neurons were not significantly different. These results suggest that duration of exposure to diazoxide is not responsible for potentiation of diazoxide currents.

We next explored the role of duration of whole-cell recording on diazoxide current augmentation. Two populations of neuron were recorded in separate slices, and diazoxide was applied either 5 min or 40 min after starting whole-cell recording. In all cases, slices had not been exposed previously to diazoxide. As shown in Fig. 5B, the initial diazoxide current was significantly larger after a 40 min delay compared to that recorded after the 5 min delay ($P < 0.01$, t test). These results show that magnitude of diazoxide current correlates with duration of whole-cell recording.

Loose patch recordings

We used loose patch recordings to avoid the intracellular dialysis that is experienced during whole-cell recording. Diazoxide (200 μ M) was superfused for 5 min every 20 min, and spontaneous firing rate of action potentials was measured before and during the final 30 sec

of each diazoxide application. As shown in Fig. 6A, diazoxide produced a modest slowing of firing rate that did not increase in magnitude with repeated applications. On average, the first application of diazoxide slowed firing rate from an initial value of 2.23 ± 0.14 Hz to 1.37 ± 0.22 Hz ($n = 5$; $P < 0.05$, paired t test), whereas the fourth application of diazoxide slowed the firing rate from 1.98 ± 0.09 Hz to 1.26 ± 0.15 Hz ($n = 5$; $P < 0.05$, paired t test). Figure 6B shows averaged data for percent inhibition of spontaneous firing rate by diazoxide. On average, repeated applications of diazoxide produced a 34% reduction in firing rate in each diazoxide trial.

We proceeded to evaluate the effect of diazoxide in the presence of A769662, which was superfused at least 1 hr before beginning loose-patch recording. However, these experiments were complicated by the fact that A769662, when superfused for one hour, caused a significant reduction in firing rate (1.39 ± 0.13 Hz; $n = 17$) compared to control ($P < 0.001$, t test). This apparent non-selective effect of A769662 to slow the spontaneous firing rate was not corrected by either dorsomorphin ($10 \mu\text{M}$) superfused for one hour ($n = 12$) or a 5-min superfusion with $100 \mu\text{M}$ tolbutamide ($n = 5$). Despite this effect of A769662 on firing rate, diazoxide had a much stronger inhibitory effect on firing rate in the presence of A769662, as shown in Fig. 7A. But most importantly, the increased inhibitory effect of diazoxide in A769662 was completely blocked by $10 \mu\text{M}$ dorsomorphin (Fig. 7A). These data, which are expressed in Fig. 7B as percentages of initial firing rates, show a significant interaction between diazoxide effect on firing rate and treatment group ($F_{(2,37)} = 12.32$, $P < 0.001$, one-way ANOVA). Diazoxide caused a $70.1 \pm 8.5\%$ reduction of initial firing rate in A769662 ($n = 17$) compared to the control value of $29.4 \pm 5.8\%$ ($n = 11$; $P < 0.01$). Moreover, pre-treatment with dorsomorphin completely prevented the potentiating effect of A769662 on diazoxide-induced inhibition of firing rate. Figure 7C shows that repeated applications of diazoxide produced approximately the same magnitude of inhibition of firing rate when recording in A769662. These data show that A769662 potentiates the inhibitory effect of diazoxide when intracellular contents are not disturbed by whole-cell recording.

Diazoxide currents using visualized patch-clamp recordings

Because all of the above studies were performed using the “blind” patch recording method, we were concerned that K-ATP current potentiation might be a phenomenon that was unique to this method. Therefore, we proceeded to perform whole-cell recordings using the visualized patch recording method. Slices for visualized recordings were identical to those used with blind patch except that slices were cut thinner than those used for blind patch recording in order to facilitate visualization of neurons (see Methods). Slices were superfused with diazoxide for 5 min every 30 min, and “augmentation” of diazoxide current was defined as at least a 50% increase in current after one hour of recording. Our experience with the blind patch method showed that 91% of SNC dopamine neurons (30 of 33) showed at least a 50% increase in diazoxide current over one hour. However, we were surprised to find that only about half of the neurons recorded under visual guidance showed potentiation of at least 50% (10 of 19). We also noted that initial diazoxide current was significantly smaller in neurons recorded with the blind patch method compared to visualized patch, as shown in Fig. 8A. We therefore evaluated visualized patch recordings for a relationship between initial diazoxide current and magnitude of current potentiation. Visualized patch

data were divided into two groups based upon initial magnitude of diazoxide current, with a cut-off of 100 pA. Of those cells in which the initial diazoxide current was less than 100 pA (average = 43 ± 5 pA), nine of 10 neurons showed potentiation of diazoxide current over one hour. In contrast, cells in which initial diazoxide current was greater than 100 pA (average = 181 ± 23 pA), only one of nine neurons showed potentiation of diazoxide current. These data, normalized to initial diazoxide current values, are shown graphically in Fig. 8B. There was a significant difference in potentiation of diazoxide current based upon magnitude of initial diazoxide current ($F_{(1,39)} = 27.22$, $P < 0.001$; two-way ANOVA).

Although we found less frequent potentiation of diazoxide currents in visualized patch recordings compared to blind patch recordings, we wondered if it was related to the magnitude of initial diazoxide current. Figure 8C shows pooled blind and visualized patch data for initial diazoxide current plotted against percent of initial diazoxide current recorded 60 min after beginning whole-cell recording. This graph shows that neurons that had relatively small initial diazoxide currents had the greatest augmentation of current over time, regardless of the type of recording platform. These data suggest that a ceiling effect may explain why neurons with high initial diazoxide current show less potentiation over time.

DISCUSSION

Results of our studies show that AMPK activation up-regulates ligand-gated K-ATP channel function in SNC dopamine neurons. Moreover, the finding that the AMPK inhibitor dorsomorphin caused rundown of diazoxide-induced currents suggests that a basal level of AMPK activity might be required for K-ATP channel function. Because potentiation of K-ATP currents under control conditions was demonstrated with whole-cell recordings but not in the loose-patch configuration, our results suggest that the process of establishing whole-cell recordings potentiates K-ATP channel function via a process involving AMPK activation. These studies show that an enzyme complex that is commonly associated with regulation of metabolic activity is important in regulation of an inhibitory K^+ current that can have a strong effect on neuronal excitability.

AMPK activation during whole-cell recording

An obvious question arises as to how the whole-cell recording process activates AMPK. Our finding that magnitude of initial diazoxide current correlates with duration of whole-cell recording suggests that dialysis of intracellular contents might play a role in AMPK activation. This conclusion is also supported by our loose-patch recordings showing that the inhibitory effect of diazoxide on firing rate remains unchanged over time, during which intracellular contents are not disturbed. Disturbance of Ca^{2+} homeostasis is one possible consequence of recording in the whole-cell configuration, and AMPK can be activated by the upstream Ca^{2+} -dependent kinase CaM-KK β (Carling et al., 2008). Another consequence of whole-cell recording is the potential rise in the AMP/ATP ratio, which is a well-known mechanism for AMPK activation (Hardie et al., 2012). However, our internal solutions contained 1.5 mM ATP, and it is difficult to understand how intracellular dialysis with ATP would increase the AMP/ATP ratio. Also, loading the cell with ATP would be expected to block K-ATP channels as opposed to causing potentiation of current (Miki and Seino, 2005).

Apart from intracellular dialysis, it is also possible that whole-cell recording could potentiate K-ATP channel function by altering the composition of the cellular membrane. For example, K-ATP channel function requires the presence of the phosphatidylinositol compound PIP-2, and increased levels of PIP-2 in the membrane facilitate K-ATP current (Shyng et al., 2000). However, it is not clear how whole-cell recording would influence membrane phospholipids via an AMPK-dependent process. Work is in progress in our lab that will investigate mechanisms for AMPK activation during whole-cell recordings.

K-ATP channel potentiation by AMPK

But once AMPK is activated, how does this potentiate K-ATP channel function? AMPK activation may facilitate the translocation of K-ATP channel subunits to the cell surface as has been shown previously in pancreatic beta-cells (Chen et al., 2013; Lim et al., 2009; Park et al., 2013b) and cardiac myocytes (Sukhodub et al., 2007). Moreover, studies in model cells have shown that AMPK-facilitated translocation of K-ATP channels occurs rapidly with as much as an 80% turnover of channels in 10-15 min (Manna et al., 2010). AMPK has also been shown to augment levels of phosphatidylinositol compounds that facilitate K-ATP channel translocation (Park et al., 2013a). Finally, AMPK has been shown to modulate K-ATP channel function via direct phosphorylation of the Kir6.2 channel subunit (Chang et al., 2009). Future studies will be needed to establish the mechanism for AMPK-dependent facilitation of K-ATP channel function in dopamine neurons.

Blind versus visualized patch recordings

One curious finding of our studies was that initial diazoxide currents were significantly larger using visualized patch recordings compared to blind patch recordings. Also, our results showed clearly that visualized patch neurons with large initial diazoxide currents showed little or no potentiation of diazoxide currents over time. One could argue that initial diazoxide currents were larger with visualized patch recording because of better access, but that would not explain the lack of potentiation of current over time. One difference between blind and visualized patch recordings is that blind patch recordings enable the recording of neurons deep in the slice, whereas visualized neurons are more likely to be recorded near the slice surface where they can be seen more clearly. Moreover, neurons recorded deep in the slice may be more likely to experience hypoxia or hypoglycemia compared to surface cells, and hypoxic/hypoglycemic conditions are well known to activate K-ATP channels in SNC neurons (Mercuri et al., 1994; Guatteo et al., 1998). Although hypoxia might be expected to cause a gradual increase in K-ATP current over time, it would not explain our finding that initial diazoxide currents were smaller in blind patch recordings compared to visualized patch recordings. An alternative explanation might be found by the fact that neurons at the slice surface are more likely to have been damaged by the process of slice preparation. One may speculate that neurons at the slice surface may have large initial K-ATP currents because AMPK has been previously been activated by cell injury. Indeed, studies have shown that traumatic brain injury can activate AMPK (Khatri and Man, 2013). It is possible that cells with pre-potentiated K-ATP currents may not show further potentiation due to a ceiling effect.

AMPK actions in neurons

Although data in neurons are limited, evidence suggests that a major function of AMPK is to conserve energy and/or to reduce neuronal excitability. For example, the AMPK activator metformin has been shown to slow the rundown of GABA_B receptor-mediated outward currents in cultured hippocampus neurons (Kuramoto et al., 2007). Furthermore, Ikematsu et al (2011) showed that AMPK activation caused a hyperpolarizing shift in the voltage dependence of current mediated Kv2.1 channels, which caused a reduction in excitatory responses of hippocampal neurons to depolarizing current pulses. AMPK activators have also been shown to inhibit glutamate-dependent long term potentiation in murine slices of hippocampus (Potter et al., 2010). In carotid body neurons, AMPK has been shown to phosphorylate TREK K⁺ channels and BK gKCa channels and reduce their conductances (Krneisz et al., 2009; Ross et al., 2011). Although these actions increase the output from carotid body neurons, the resulting stimulation of medullary center increases respiratory drive and thereby facilitates oxidative phosphorylation. One of the better documented effects of AMPK activation involves regulation of appetite via actions in the hypothalamus. Thus, AMPK activation has been shown to trigger Ca²⁺ influx and increase cFOS expression in neuropeptide Y neurons in the arcuate nucleus, which has the effect of promoting feeding behavior in rats (Kohno et al., 2011; Dickson and Luckman, 1997). Moreover, the orexigenic hormone ghrelin stimulates appetite by an action dependent upon AMPK phosphorylation in arcuate neurons (Kirsch and Zieba, 2011). Furthermore, hypoglycemia has been shown to increase K-ATP current via a process that requires AMPK activity in glucose-sensing neurons of the mouse hypothalamus, which is essential for maintaining appetite (Beall et al., 2010; Claret et al., 2007). Finally, our lab reported recently that AMPK augments K-ATP current evoked by NMDA receptor stimulation in STN neurons, whereas ligand-gated K-ATP current was not affected (Shen and Johnson, 2010). Moreover, NMDA did not induce K-ATP current in SNC dopamine neurons (Shen and Johnson, 2010). In contrast to findings in the STN, the present study demonstrated a gradual potentiation of ligand-gated K-ATP currents when AMPK is activated during whole-cell recordings in the SNC. These studies show that actions of AMPK may differ depending upon type of neuron. However, these data are also consistent with those done in somatic tissues showing that AMPK plays a conservative role to reduce neuronal excitability and/or to maintain energy homeostasis.

AMPK actions in SNC neurons

The role of AMPK to dampen neuronal excitability may be especially applicable to SNC dopamine neurons. As opposed to other neuronal types, the pacemaker mechanism that maintains spontaneous activity in SNC neurons relies upon rhythmic Ca²⁺ influx through L-type channels (Chan et al., 2007), and this Ca²⁺ dependence has been postulated to increase the vulnerability of SNC dopamine neurons to cell death in Parkinson's disease (Dryanovski et al., 2013). Moreover, the firing of action potentials is metabolically expensive because Ca²⁺ and Na⁺ that enter during action potentials must be extruded by ATP-consuming exchangers in order to maintain ionic homeostasis. The hyperpolarizing influence of K-ATP current reduces spike frequency and, therefore, the need to consume ATP to maintain homeostasis is reduced. Results of our present study suggest that K-ATP currents will intensify when metabolic conditions activate AMPK, and this should reduce the need for

energy consumption. Our results are consistent with the hypothesis that AMPK acts to promote the conservation of metabolic energy and/or dampen excitability in central neurons.

CONCLUSIONS

Our studies show that AMPK activity facilitates the hyperpolarizing influence of K-ATP current in SNC dopamine neurons. Moreover, our finding that an AMPK inhibitor reduced K-ATP current suggests that a basal level of AMPK activity may be necessary to maintain K-ATP channel function. We suggest that augmentation of K-ATP channel function is consistent with a global hypothesis that AMPK plays an important role to conserve metabolic energy and/or reduce neuronal excitability.

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Abbreviations

AMPK	AMP-activated protein kinase
K-ATP channel	ATP-sensitive K ⁺ channel
NMDA	N-methyl-D-aspartate
SNC	Substantia nigra zona compacta
STN	Subthalamic nucleus
SUR	Sulfonylurea binding protein

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Highlights

- AMP kinase (AMPK) and K-ATP channels both respond to alterations in metabolic conditions
- We used patch clamp recordings to study AMPK/K-ATP interactions in rat substantia nigra compacta (SNC) dopamine neurons
- K-ATP current was increased by AMPK activation and reduced by an AMPK inhibitor
- Duration of recording in whole-cell configuration correlated with magnitude of K-ATP current
- AMPK regulates neuronal excitability by facilitating K-ATP channel function in SNC neurons

Fig. 1.

Diazoxide currents augment over time via an AMPK-dependent mechanism. A) Currents evoked by repeated applications of diazoxide (200 μ M) increase over time. Recording also shows that current was completely blocked by tolbutamide (100 μ M). Dashed line indicates zero current. B) Summary of diazoxide-induced currents over time. Graph shows that currents evoked by repeated applications of diazoxide increase over time. Pipettes contained normal internal solution. Each data point is the mean of 8-16 neurons. C) Summary graph showing diazoxide currents over time when recorded with A769662, with normal pipette solution (control), and when slices were superfused with dorsomorphin (20 μ M). A769662 was either added to the pipette solution (5 μ M) or the superfusate (30 μ M). Data were normalized to initial diazoxide current for each cell. Each data point represents 6-16 neurons. Asterisks indicate significant differences compared to control values (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Sidak pairwise tests).

Fig. 2.

Diazoxide conductance increases with repeated applications. A) Current traces recorded during a series of hyperpolarizing voltage steps. The slice was superfused with diazoxide (200 μ M) for five min with an interval of 20 min. Recordings are from the same neuron. B) Average current-voltage plots for the first and second applications of diazoxide. Paired data are from 5 neurons. Asterisks indicate significant differences compared to control values (*, $P < 0.05$; paired t tests with Bonferroni correction).

Fig. 3.

Tolbutamide-sensitive currents augment over time in the presence of the K-ATP channel opener NN-414. A) Current trace recorded in the presence of NN-414 (10 μM). Because effects of NN-414 did not washout readily, K-ATP currents were measured by repeatedly superfusing tolbutamide (100 μM). B) Summary graph showing that tolbutamide-sensitive currents increase over time in the presence of NN-414. Each data point is the mean of 4 neurons. Asterisk indicates significant difference compared to the initial tolbutamide-sensitive current (*, $P < 0.05$; Sidak pairwise test).

Fig. 4. A769662 fails to augment baclofen-induced currents. A) Current trace showing effects of repeated applications of baclofen (1 μ M). Pipette contained A769662 (5 μ M). B) Averaged data showing gradual run-down of baclofen-induced currents over time when recorded in the presence of A769662. Each data point represents the average response of 5-7 neurons.

Fig. 5.

Effects of prior diazoxide exposure and duration of whole-cell recording on diazoxide currents. A) Augmentation of current is not dependent upon prior diazoxide exposure. Two neurons were recorded sequentially in each brain slice. Both the first and second neurons recorded show significant augmentation of diazoxide current despite the fact that second neurons had been exposed previously to repeated applications of diazoxide. Data are averages from 5-9 neurons. Asterisks indicate significant differences compared to the initial diazoxide current (*, $P < 0.05$; **, $P < 0.01$; Sidak pairwise tests). B) Augmentation of diazoxide current is dependent upon duration of whole-cell recording. Diazoxide was superfused after a delay of either 5 or 40 min after beginning whole-cell recording. Data represent two separate populations of neuron ($n = 5$ for each group), and each slice had not been superfused previously with diazoxide. Asterisks indicate significance (**, $P < 0.01$, t test).

Fig. 6.

Inhibitory action of diazoxide fails to increase over time when recording in loose-patch configuration. A) Voltage traces show mild inhibitory effects on spontaneous firing of action potentials in a SNC dopamine neuron. Diazoxide (200 μ M) was superfused for 5 min every 20 min, and firing rate was measured over the final 30 sec of each drug application. The inhibitory effect of the first application of diazoxide was not different from that observed during the fourth application. B) Summary graph shows percent inhibition of firing rate by diazoxide. Firing rate was reduced by about 34% during each application of diazoxide ($n = 5$).

Fig. 7.

AMPK facilitates diazoxide effect in loose patch recordings. A) Voltage traces show that diazoxide exerts stronger inhibition of firing rate in the presence of A769662. The inhibitory effect of diazoxide (200 μ M) in A769662 (10 μ M) is reduced by dorsomorphin (10 μ M). Each of the three sequences is from a different dopamine neuron. B) Averaged data show that diazoxide produced significantly greater inhibition of firing in A769662 compared to control, and this effect was prevented by dorsomorphin. Both A769662 and dorsomorphin were perfused at least 1 hr prior to superfusion with diazoxide. Data were normalized to the initial firing rate of each neuron in each treatment group. Asterisk indicates a significant difference compared to the control group (**, $P < 0.01$; Sidak pairwise test). C) Repeated applications of diazoxide produce comparable reductions in firing rate when recording in A769662 ($n = 3$).

Fig. 8.

Potentiation of diazoxide current is dependent upon magnitude of initial diazoxide current.

A) Initial diazoxide current is larger in neurons recorded in visualized patch recordings ($n = 19$) compared to blind patch recordings ($n = 39$; **, $P < 0.01$, t test). B) Potentiation of diazoxide current occurs when initial diazoxide current is less than 100 pA. Neurons in visualized patch recordings were divided into groups in which initial diazoxide current was either less than 100 pA ($n = 10$) or greater than 100 pA ($n = 9$). Data were normalized to magnitude of initial diazoxide current. Asterisks indicate significant differences between groups (*, $P < 0.05$; ***, $P < 0.001$; Sidak pairwise test). C) Pooled data from blind and visualized patch recordings showing the correlation between magnitude of initial diazoxide current and percent of initial diazoxide current 60 min after starting whole-cell recordings. Curve represents data fitted to the logarithm of initial diazoxide current ($R = 0.691$).